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(71) Applicant *(for all designated States except US)*: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE [BE/BE]; Rijvisschestraat 120, B–9052 Zwijnaarde (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LEE, Jeong, Hee [KR/BE]; Spinmolenplein 274 (22K), B–9000 Gent (BE). VERBRUGGEN, Nathalie [BE/BE]; Avenue des Saisons, 53, B–1050 Ixelles (BE).

(74) Agent: DE CLERCQ, Ann; Ann De Clercq & Co. B.V.B.A., Brandstraat 100, B–9830 Sint–Martens–Latem (BE).

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(57) Abstract

The present invention relates to a method for obtaining polynucleic acids comprising coding sequences and/or genes involved in environmental stress resistance in plants, comprising the preparation of a cDNA library comprising coding sequences from siliques, introducing said coding sequences in yeast cells in a functional format and screening for polynucleic acids leading to an enhanced tolerance or resistance to environmental stress conditions in said transformed yeast cells. The present invention further relates to an isolated polynucleic acid obtainable by such a method as listed in Table 1 as well as recombinant polynucleic acid comprising the same. The present invention further relates to an isolated polypeptide encoded by a polynucleic acid of the invention. The present invention also relates to a method for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into a plant cell a recombinant DNA comprising a polynucleic acid as defined which when expressed in a plant cell enhances the tolerances or induces resistance to environmental stress conditions of said plant. The present invention particularly relates to plant cells, plants or harvestable parts or propagation material thereof transformed with a recombinant polynucleic acid as defined above.

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Genes involved in t lerance to nvironmental str ss

The present invention relates to molecular biology, in particular plant molecular biology. In particular, the invention relates to improvements of crop productivity of useful plants. One of the major limitations of crop productivity is the effect of environmental stress conditions on plant growth and development. An important goal of molecular biology is the identification and isolation of genes that can provide resistance or tolerance to such stresses. For agriculture, the creation of transgenic plants containing such genes provides the potential for improving the stress resistance or tolerance of plants.

Drought, salt loading, and freezing are stresses that cause adverse effects on the growth of plants and the productivity of crops. The physiological response to these stresses arises out of changes in cellular gene expression. Expression of a number of genes has been demonstrated to be induced by these stresses (Zhu et al., 1997; Shinozaki et al., 1996; Thomashow, 1994). The products of these genes can be classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response. The first group includes proteins that likely function by protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes (Shinozaki et al., 1997, Ingram et al., 1996, Bray et al., 1997). The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism (Shinozaki et al., 1997). An overview of the methods known to improve stress tolerance in plants is also given in Holmberg & Bülow, (1998).

Further studies are definitely needed to give an insight into the mechanisms involved in the plant response to environmental stress conditions.

The study of plants naturally adapted to extreme desiccation has led to the hypothesis that the genetic information for tolerance to environmental stress conditions exists in all higher plants. In glycophytes, this information would only be expressed in seeds and pollen grains which undergo a desiccation process.

The induction of osmotolerance in plants is very important to crop productivity: 30 to 50 % of the land under irrigation is presently affected by salinity. Several lines of evidence also demonstrate that even mild environmental stress conditions throughout the growth season have a negative impact on plant growth and crop productivity. It is

for instance known that even minor limitations in water availability cause a reduced photosynthetic rate. Unpredictable rainfall, increase in soil salinity at the beginning and the end of the growing season often result in decreased plant growth and crop productivity. These environmental factors share at least one element of stress and that is water deficit or dehydration. Drought is a significant problem in agriculture today. Over the last 40 years, for example, drought accounted for 74% of the total US crop losses of corn. To sustain productivity under adverse environmental conditions, it is important to provide crops with a genetic basis for coping with water deficit, for example by breeding water retention and tolerance mechanisms into crops so that they can grow and yield under these adverse conditions.

It is an aim of the present invention to provide a new method for screening for plant genes involved in tolerance or resistance to environmental stress.

It is an aim of the present invention to provide new plant genes, more particularly plant genes providing the potential of improving the tolerance to environmental stress conditions in plants.

It is also an aim of the present invention to provide polypeptides encoded by said new plant genes.

It is further an aim of the present invention to provide methods for producing plants with enhanced tolerance or resistance to environmental stress conditions based on said new genes.

It is also an aim of the present invention to provide recombinant polynucleic acids comprising said new genes.

It is further an aim of the present invention to provide plant cells and plants transformed with said new genes.

It is further an aim of the present invention to provide plant cells and plants with enhanced tolerance or resistance to environmental stress conditions.

The present invention relates more particularly to a method for obtaining polynucleic acids comprising coding sequences and/or genes involved in environmental stress in plants, comprising the preparation of a cDNA library comprising coding sequences from siliques, introducing said coding sequences in yeast cells in a functional format and screening for polynucleic acids leading to an enhanced tolerance or resistance to environmental stress conditions in said transformed yeast cells.

It has been found that the transfer of genes from plants which are often difficult to assay for certain characteristics, to lower eukaryotes, such as yeasts and fungi, but

in particular yeast, especially Saccharomyces, is relatively-easy to achieve, whereby it has now been shown that the results of testing for tolerance or resistance to environmental conditions in the resulting yeast cells gives a relatively reliable measure of the capability of the inserted coding sequence or gene to induce tolerance or resistance to environmental stress in plants. Thus the expression of polynucleic acid sequences comprising the gene or coding sequence which are responsible for inducing tolerance or resistance to environmental stress conditions can be enhanced in the plant species from which it originates or in any other plant species.

In the present context the term "enhancing" must be understood to mean that the levels of molecules correlated with stress protection in a transformed plant cell, plant tissue or plant part will be "substantially increased" or "elevated" meaning that this level will be greater than the levels in an untransformed plant.

This may be achieved by inducing overexpression of suitable genetic information which is already present, or by any other suitable means of introducing into the plant cell heterologous information resulting in a capability to tolerate or resist environmental stress.

The term "environmental stress" has been defined in different ways in the prior art and largely overlaps with the term "osmotic stress". Holmberg et al., 1998 for instance define different environmental stress factors which result in abiotic stress. Salinity, drought, heat, chilling and freezing are all described as examples of conditions which induce osmotic stress. The term "environmental stress" as used in the present invention refers to any adverse effect on metabolism, growth or viability of the cell, tissue, seed, organ or whole plant which is produced by an non-living or non-biological environmental stressor. More particularly, it also encompasses environmental factors such as water stress (flooding, drought, dehydration), anaerobic (low level of oxygen, CO_2 etc.), aerobic stress, osmotic stress, salt stress, temperature stress (hot/heat, cold, freezing, frost) or nutrients/pollutants stress.

The term "anaerobic stress" means any reduction in oxygen levels sufficient to produce a stress as hereinbefore defined, including hypoxia and anoxia.

The term "flooding stress" refers to any stress which is associated with or induced by prolonged or transient immersion of a plant, plant part, tissue or isolated cell in a liquid medium such as occurs during monsoon, wet season, flash flooding or excessive irrigation of plants, etc.

"Cold stress" and "heat stress" are stresses induced by temperatures which are respectively, below or above, the optimum range of growth temperatures for a

particular plant species. Such optimum growth temperature ranges are readily determined or known to those skilled in the art.

"Dehydration stress" is any stress which is associated with or induced by the loss of water, reduced turgor or reduced water content of a cell, tissue, organ or whole plant.

"Drought stress" refers to any stress which is induced by or associated with the deprivation of water or reduced supply of water to a cell, tissue, organ or organism.

"Oxidative stress" refers to any stress which increases the intracellular level of reactive oxygen species.

The terms "salinity-induced stress", "salt-stress" or similar term refer to any stress which is associated with or induced by elevated concentrations of salt and which result in a perturbation in the osmotic potential of the intracellular or extracellular environment of a cell.

Said salt can be for example, water soluble inorganic salts such as sodium sulfate, magnesium sulfate, calcium sulfate, sodium chloride, magnesium chloride, calcium chloride, potassium chloride etc., salts of agricultural fertilizers and salts associated with alkaline or acid soil conditions.

The transgenic plants obtained in accordance with the method of the present invention, upon the presence of the polynucleic acid and/or regulatory sequence introduced into said plant, attain resistance, tolerance or improved tolerance or resistance against environmental stress which the corresponding wild-type plant was susceptible to.

The terms "tolerance" and "resistance" cover the range of protection from a delay to complete inhibition of alteration in cellular metabolism, reduced cell growth and/or cell death caused by the environmental stress conditions defined herein before. Preferably, the transgenic plant obtained in accordance with the method of the present invention is tolerant or resistant to environmental stress conditions in the sense that said plant is capable of growing substantially normal under environmental conditions where the corresponding wild-type plant shows reduced growth, metabolism, viability, productivity and/or male or female sterility. Methodologies to determine plant growth or response to stress include, but are not limited to height measurements, leaf area, plant water relations, ability to flower, ability to generate progeny and yield or any other methodology known to those skilled in the art.

The terms "tolerance" and "resistance" may be used interchangeably in the present invention.

The methods according to the invention as set out below can be applied to any, higher plant, preferably important crops, preferably to all cells of a plant leading to an enhanced osmotic or any other form of environmental stress tolerance. By means of the embodiments as set out below, it now becomes possible to grow crops with improved yield, growth, development and productivity under environmental stress conditions, it may even become possible for instance to grow crops in areas where they cannot grow without the induced osmotolerance according to the invention.

In order to do a thorough screening for relevant plant genes and/or coding sequences, it is preferred to apply a method according to the invention whereby said cDNA library comprises copies of essentially all mRNA of said plant cell. Probably only coding sequences are sufficient. For the screening of genes involved in environmental stress, it is preferred to use a cDNA library from siliques (fruits, containing the maturing seeds), such as the siliques from *Arabidopsis*, because genes involved in for instance osmotolerance are preferentially expressed in these organs.

Although the genetic information may be introduced into yeast for screening by any suitable method, as long as it is in a functional format long enough tor testing of tolerance or resistance to environmental stress conditions, it is preferred for ease of operation to use a well known vector such as a 2μ plasmid. It is to be preferred to have the coding sequence or the gene under control of a strong constitutive yeast promoter, to enhance good expression of the gene or coding sequence of interest. Strong constitutive yeast promoters are well known in the art and include, but are not limited to the yeast TPI promoter.

The term "gene" as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter and a 5' untranslated region (the 5'UTR), a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. Typically in plant cells, the 5'UTR, the coding region and the 3'UTR (together referred to as the transcribed DNA region) are transcribed into an RNA which, in the case of a protein encoding gene, is translated into a protein. A gene may include additional DNA fragments such as, for example, introns. As used herein, a genetic locus is the position of a given gene in the genome of a plant.

The present invention more particularly relates to an isolated polynucleic acid obtainable by a method comprising the preparation of a cDNA as set out above comprising coding sequences from siliques, introducing said coding sequences in yeast cells in a functional format and screening for polynucleic acids leading to an

enhanced tolerance or resistance to environmental stress conditions in said transformed yeast cells.

The term "polynucleic acid" refers to DNA or RNA, or amplified versions thereof, or the complement thereof.

The present invention more particularly provides an isolated polynucleic acid obtainable by a method as defined above which encodes a polypeptide as listed in Table 1.

The capacity of an isolated polynucleic acid to confer tolerance or resistance to environmental stress conditions can be tested according to methods well-known in the art, see for example, Grillo et al. (1996), Peassarakli et al. (Editor), Nilsen et al. (1996), Shinozaki et al. (1999), Jones et al. (1989), Fowden et al. (1993) or as described in the appended examples.

The present invention more particularly relates to an isolated polynucleic acid which encodes a homolog of any of the polypeptides as listed in Table 1, which is chosen from:

(a) any of SEQ ID NO 1, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 121, or the complementary strands thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b), or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

Preferably said sequences according to part (b) hybridize under stringent conditions to the sequences of part (a).

Said fragment as defined above are preferably unique fragments of said sequences.

The term "hybridizing" refers to hybridization conditions as described in Sambrook (1989), preferably specific or stringent hybridization conditions are aimed at.

Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least about 60°C.

In the present invention, genomic DNA or cDNA comprising the polynucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the cDNA sequence shown. The preparation of both genomic and cDNA libraries is within the skill of the art. Examples of hybridization conditions are also given in the Examples section.

The present invention also relates to the isolated polynucleic acids which encode polypeptides which are a homolog of the polypeptides as set out in Table 1 useful for the production of plants which are resistant or tolerant to environmental stress conditions.

The present invention also relates to a polynucleic acid comprising at least part of any of SEQ ID NO 1, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77 or 121, or at least part of a gene that is at least 50% identical, preferentially at least 55%, 60%, 65% or 70% identical, more preferably at least 75%, 80% or 85% identical, and most preferably at least 90% or 95% identical to any of SEQ ID NO 1, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77 or 121. Preferably, said gene encodes a protein having substantially the same biological activity as the protein having the sequence of SEQ ID NO 2, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76 or 78. Said part of said gene is preferably a unique part.

The present invention preferably relates to the use of a polynucleic acid comprising at least part of any of SEQ ID NO 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, or 121, or at least part of a gene that is at least 50% identical, preferentially at least 55%, 60%, 65% or 70% identical, more preferably at least 75%, 80% or 85% identical, and most preferably at least 90% or 95% identical to any of

SEQ ID NO 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, or 121 for the production of transgenic plants having enhanced tolerance or resistance to environmental stress conditions.

Preferably, said gene encodes a protein having substantially the same biological activity as the protein having the sequence of SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, or 120. Said part of said gene is preferably a unique part.

The present invention particularly relates to an isolated polynucleic acid as defined above, which encodes a plant homolog of yeast DBF2 kinase, more particularly a DBF2 kinase homolog from Arabidopsis thaliana termed At-DBF2, which can at least be used to confer enhanced environmental stress tolerance or resistance in plants and yeast.

More preferably, the present invention relates to an isolated polynucleic acid encoding a plant DFB2 kinase, which is chosen from:

(a) SEQ ID NO 1, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(e) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b), or,

(c) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

Preferably said sequences according to part (b) hybridize under stringent conditions to the sequences of part (a).

Alternatively, the present invention relates to a polynucleic acid derived from a plant comprising at least part of SEQ ID NO 1, or at least part of a gene having a sequence that is at least 50% identical, preferentially at least 55%, 60%, 65% or 70% identical, more preferably at least 75%, 80% or 85% identical, and most preferably at least 90% or 95% identical to SEQ ID NO 1. Preferably said gene encodes a protein

having substantially the same biological activity as the protein having the sequence of SEQ ID NO 2.

The present invention also relates to the use of an isolated polynucleic acid as defined above which encodes a plant HSP 17.6A protein for the production of transgenic plants, more particularly a homolog from Arabidopsis thaliana, which at least can be used to confer enhanced environmental stress tolerance in plants and yeast.

More preferably, the present invention relates to the use of an isolated polynucleic acid as defined above which is chosen from:

(a) SEQ ID NO 3, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b) or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c),

for the production of transgenic plants having an enhanced tolerance or resistance to environmental stress conditions.

Preferably said sequences according to part (b) hybridize under stringent conditions to the sequences of part (a).

The present invention also relates to the use of a polynucleic acid comprising at least part of SEQ ID NO 3, or at least part of a gene having a sequence that is at least 50% identical, preferentially at least 55%, 60%, 65% or 70% identical, more preferably at least 75%, 80% or 85% identical, and most preferably at least 90% or 95% identical to SEQ ID NO 3. Preferably said gene encodes a protein having substantially the same biological activity as the protein having the sequence of SEQ ID NO 4, for the production of transgenic plants having enhanced tolerance or resistance to environmental stress conditions.

More preferably, the present invention relates to the use of an isolated polynucleic acid as defined above which is chosen from:

(a) any of SEQ ID NO 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, or 119, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b) or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c),

for the production of transgenic plants having an enhanced tolerance or resistance to environmental stress conditions.

The present invention preferably relates to the use of a polynucleic acid comprising at least part of any of SEQ ID NO 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, or 119, or at least part of a gene that is at least 50% identical, preferentially at least 55%, 60%, 65% or 70% identical, more preferably at least 75%, 80% or 85% identical, and most preferably at least 90% or 95% identical to any of SEQ ID NO 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, or 119, for the production of transgenic plants having enhanced tolerance or resistance to environmental stress conditions.

Preferably, said gene encodes a protein having substantially the same biological activity as the protein having the sequence of SEQ ID NO 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, or 120. Said part of said gene is preferably a unique part.

According to another preferred embodiment, the present invention relates to an isolated polynucleic acid as defined above, which encodes a protein termed c74, more particularly a plant homolog of c74, even more preferably a c74 from Arabidopsis thaliana, which at least can be used to confer enhanced environmental stress tolerance in plants and yeast.

More particularly, the present invention relates to an isolated polynucleic acid as defined above, which is chosen from:

(a) SEQ ID NO 5, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b) or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

Preferably said sequences according to part (b) hybridize under stringent conditions to the sequences of part (a).

The present invention also relates to a polynucleic acid comprising at least part of SEQ ID NO 5, or at least part of a gene having a sequence that is at least 50% identical, preferentially at least 55%, 60%, 65% or 70% identical, more preferably at least 75%, 80% or 85% identical, and most preferably at least 90% or 95% identical to SEQ ID NO 5. Preferably said gene encodes a protein having substantially the same biological activity as the protein having the sequence of SEQ ID NO 6.

Two nucleic acid sequences or polypeptides are said to be "identical" according to the present invention if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the complementary sequence hybridizes to all or a portion of a given polynucleotide sequence.

Sequence comparisons between two (or more) polynucleic acid or polypeptide sequences are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsch (1970), by the search for similarity method of Pearson and Lipman (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by visual inspection.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleic acid or polypeptide sequences in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of polynucleic acid or polypeptide sequences means that a polynucleotide sequence comprises a sequence that has at least 60%, 65%, 70% or 75% sequence identity, preferably at least 80% or 85%, more preferably at least 90% and most preferably at least 95 %, compared to a reference sequence using the programs described above (preferably BLAST) using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 40%, 45%, 50% or 55% preferably at least 60%, 65%, 70%, 75%, 80% or 85% more preferably at least 90%, and most preferably at least 95%. Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other, or a third nucleic acid, under stringent conditions.

More particularly, the polynucleic acids as used herein will comprise at least part of a DNA sequence which is essentially similar, or, preferentially, essentially identical or identical to one or both of the nucleotide or amino acid sequences corresponding to SEQ ID NO 1 to 121 disclosed herein, more specifically in the nucleotide sequence encoding, or the amino-acid sequence corresponding to the "active domain" of the respective protein or polypeptide.

The polynucleic acid sequences according to the present invention can be produced by means of any nucleic acid amplification technique known in the art such as PCR or conventional chemical synthesis.

For a general overview of PCR see PCR Protocols (Innis et al. (1990)).

Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers et al. (1982) and Adams et al. (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The present invention more particularly relates to an isolated polypeptide encoded by a polynucleic acid according to any of the polynucleic acids as defined above, or a functional fragment thereof.

The present invention preferably relates to an isolated polypeptide as listed in Table 1 or to an isolated polypeptide encoded by a polynucleic acid isolated as defined above. Preferably, the present invention relates to polypeptides or peptides having at least part of the sequence of any of SEQ ID NO NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, or 120. Preferably, said part is a unique part and preferably includes the active domain of said polypeptide. Preferably said polypeptide is a recombinant polypeptide.

The term "isolated" distinguishes the protein or polynucleic acid according to the invention from the naturally occuring one.

The present invention also relates to a polypeptide comprising at least part of a polypeptide which is at least 50%, 55%, 60%, 65% identical, preferentially at least 70%, 75% identical, more preferably at least 80% or 85% identical, and most

preferably at least 90% or 95% identical to any of SEQ ID NO NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, or 120.

The terms "polypeptide" and "protein" are used interchangeably throughout the present description.

Said polypeptide preferably has the ability to confer tolerance or resistance to environmental stress conditions in at least plants, plant parts, plant tissues, plant cells, plant calli or yeast.

The term "functional fragment" refers to a fragment having substantially the biological activity of the protein from which it is derived.

The polypeptides of the present invention may be produced by recombinant expression in prokaryotic and eukaryotic engineered cells such as bacteria, yeast or fungi. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression in these systems.

The present invention more particularly relates to a method for producing a plant with enhanced environmental stress resistance or tolerance, said method comprising transiently introducing into a plant cell a recombinant DNA comprising any of the polynucleic acids as defined above which when (over)expressed in a plant cell enhances tolerance or resistance to environmental stress of said plant.

The term "plant cell" as defined above also comprises plant tissue or a plant as a whole. The present invention more particularly relates to a method for producing a plant with enhanced environmental stress resistance or tolerance, said method comprising transiently introducing into a plant cell a recombinant DNA comprising any of the polynucleic acids encoding a protein as listed in Table 1 which when (over)expressed in a plant cell enhances tolerance or resistance to environmental stress in said plant.

The term "(over)expression" refers to the fact that the polypeptides of the invention encoded by said polynucleic acid are preferably expressed in an amount effective to confer tolerance or resistance to the transformed plant, to an amount of salt, heat, cold, (or other stress factors) that inhibits the growth of the corresponding untransformed plant.

Several methods to obtain transient introduction and expression of a recombinant DNA in a plant are known to the art. For example, plant virus vectors can

be used to obtain such purpose. Examples conferring to the use of plant viral vectors are described in Porta and Lomonossoff (1996), WO9320217 and US 5,589,367.

The present invention also relates to a method for producing a plant with enhanced environmental stress resistance or tolerance, said method comprising stably introducing into the genome of a plant cell a recombinant DNA comprising any of the polynucleic acids as defined above which when (over)expressed in a plant cell enhances the environmental stress tolerance or resistance of a plant.

The present invention also relates to a method for producing a plant with enhanced tolerance or resistance to environmental stress conditions, said method comprising introducing into the genome of a plant cell a recombinant DNA comprising any of the polynucleic acids encoding a protein as listed Table 1 which when (over)expressed in a plant cell enhances the environmental stress resistance of said plant.

According to a preferred embodiment, the present invention relates to a method for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into said plant a polynucleic acid as defined above encoding a DBF2 kinase, preferably a plant DBF2 kinase, most preferably an Arabidopsis DBF2 kinase.

According to another preferred embodiment, the present invention relates to a method as defined above for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into said plant a polynucleic acid as defined above encoding an HSP 17.6A protein, preferably a plant HSP 17.6A protein, most preferably an Arabidopsis HSP 17.6A.

According to a preferred embodiment, the present invention relates to a method as defined above for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into said plant a polynucleic acid as defined above encoding a c74 protein, preferably a plant c74 protein, most preferably a Arabidopsis c74 protein.

Preferably, the present invention relates to a method as defined above, comprising:

(a) introducing into the genome of a plant cell one or more recombinant DNA molecules, said recombinant DNA molecules comprising:
   - a polynucleic acid as defined above, and,
   - a plant expressible promoter, whereby said polynucleic acid is in the same transcriptional unit

and under the control of said plant-expressible promoter, and,

(b) regenerating said plant from said plant cell.

The present invention also relates to a method for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising indirectly increasing of inducing the expression of an endogenous gene in said plant comprised within a polynucleic acid as defined above or indirectly increasing of inducing te activity of a protein as defined above.

The present invention also relates to a method as defined above, comprising:

(a) introducing into the genome of a plant cell one or more recombinant DNA molecules, said recombinant DNA molecules comprising:

- a DNA encoding a protein which when expressed in said plant cell at an effective amount indirectly increases or induces the expression of an endogenous polynucleic acid or indirectly increases or induces the protein activity of a protein encoded by said polynucleic acid of the present invention, and,
- a plant expressible promoter, whereby said DNA is in the same transcriptional unit and under the control of said plant-expressible promoter, and,

(b) regenerating said plant from said plant cell.

A "recombinant" DNA molecule will comprise a "heterologous sequence" meaning that said recombinant DNA molecule will comprise a sequence originating from a foreign species, or, if from the same species, may be substantially modified from its original form. For example, a promoter operably linked to a structural gene which is from a species different from which the structural gene was derived, or, if from the same species, may be substantially modified from its original form.

The present invention also relates to a method as defined above for producing a plant with enhanced tolerance or resistance to environmental stress conditions, said method comprising indirectly increasing or inducing the expression of an endogenous gene in said plant comprised within a polynucleic acid as defined above or indirectly increasing or inducing the activity of a protein of the invention as defined above. According to this embodiment, other polynucleic acids modulating the expression or the activity of a protein according to the present invention may be introduced

transiently or stably into the genome of said plants. The term "modulating" means enhancing, inducing, increasing, decreasing or inhibiting.

Increase or induction of expression or induction or increase of protein activity is required when said regulator protein is a positive regulator of the expression or the activity of at least one of the polynucleic acids or protein of the present invention.

Decrease or inhibition of expression or decrease or inhibition of protein activity is required when said regulator protein is a negative regulator of the expression or activity of at least one of the polynucleic acids or proteins of the present invention.

Increase of the activity of said polypeptide according to the present invention is obtained, according to one embodiment of the invention, by influencing endogenous gene expression in the plant. This is preferably achieved by the introduction of one or more polynucleic acid sequences according to the invention into the plant genome, in a suitable conformation for gene expression (e.g. under control of a plant-expressible promoter). This will result in increased or induced expression (overexpression) or increased or induced activity of the protein in the plant cells, and, in the presence of an adequate substrate, in an increase of tolerance or resistance to environmental stress conditions in a transgenic plant or plant cell as compared to a non-transgenic plant or plant cell. This increase in tolerance can be measured by measuring mRNA levels, or where appropriate, the level or activity of the respective protein (e.g. by means of ELISA, activity of the enzyme as measured by any technique known in the art). Endogenous gene expression refers to the expression of a protein which is naturally found in the plant, plant part or plant cell concerned.

Alternatively, said enhanced tolerance or resistance to environmental stress conditions may be achieved by introducing into the genome of the plant, one or more transgenes which interact with the expression of endogenous genes (polynucleic acids) according to the present invention, by anti-sense RNA, co-suppression or ribozyme suppression of genes which normally inhibit the expression of the polynucleic acids of the present invention or by suppression of genes which normally inhibit the activity of the polypeptides of the invention as defined above.

For inhibition of expression, the nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other genes within a family of genes exhibiting homology or substantial homology to the target gene.

For antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA.

Generally, higher homology can be used to compensate for the use of a shorter sequence.

Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides up to the full length sequence should be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of about 500 to about 1700 nucleotides is especially preferred.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of genes as explained above. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs which are capable of selfcleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Haseloff et al. (1988).

Another method of suppression of gene expression is sense suppression. Introduction of nucleic acid configured in the sense orientation has been shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al. (1990), and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184.

The suppressive effect may occur where the introduced sequence contains no coding sequence per se, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous

sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

For sense suppression, the introduced sequence, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed mRNA. This may be preferred to avoid concurrent production of some plants which are overexpressers. A higher identity in a shorter than full length sequence compensates for a longer, less identical sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted above for antisense regulation is used.

Other methods for altering or replacing genes known in the art can also be used to inhibit expression of a gene. For instance, insertional mutants using T-DNA or transposons can be generated. See, e.g., Haring et al. (1991) and Walbot (1992). Another strategy in genetic engineering of plants and animals is targeted gene replacement. Homologous recombination has typically been used for this purpose (see, Capecchi (1989)).

Alternatively, the present invention also relates to a method as defined above wherein said DNA encodes a sense or antisense RNA or a ribozyme capable of indirectly increasing or inducing the expression of an endogenous polynucleic acid sequence according to the invention as defined above or increasing or inducing the activity of a protein of the invention as defined above. Preferably said endogenous polynucleic acid encodes a protein as listed in Table 1.

The present invention also relates to a recombinant polynucleic acid comprising: a polynucleic acid as defined above, and, a plant expressible promoter, whereby said polynucleic acid is in the same transcriptional unit and under the control of said plant-expressible promoter.

The present invention also relates to a recombinant polynucleic acid comprising:

(a) a DNA encoding a protein which when expressed in said plant at an effective amount indirectly increases or induces the expression of an endogenous

polynucleic acid as defined above or indirectly increases or induces the protein activity of a polypeptide as defined above, and,

(b) a plant expressible promoter, whereby said DNA is in the same transcriptional unit and under the control of said plant-expressible promoter.

An "endogenous" polynucleic acid refers to a polynucleic acid that is already present in the plant species before transformation.

Said recombinant polynucleic acid as described here above is generally also referred to as a "recombinant vector" or an "expression cassette". An expression cassette of the invention can be cloned into an expression vector by standard methods. The expression vector can then be introduced into host cells by currently available DNA transfer methods.

The present invention also relates to the recombinant polynucleic acid as defined above, comprising a DNA which encodes an anti-sense RNA, a ribozyme or a sense RNA which increases or induces the activity of a protein as defined above in said cell. Preferably said protein is listed in Table 1.

More particularly, the present invention relates to a recombinant polynucleic acid comprising at least part of the nucleotide sequence of any of SEQ ID NO 1, 3, 5, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, or 121.

Preferably, the present invention relates to a recombinant polynucleic acid comprising at least part of the coding sequence of a gene encoding a protein as listed in Table 1. Preferably, said "part" is a unique part of any of said nucleotide sequences. (26-28) As used herein, the term a "plant-expressible promoter" refers to a promoter that is capable of driving transcription in a plant cell. This includes any promoter of plant origin, including the natural promoter of the transcribed DNA sequence, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell. The promoter may also be an artificial or synthetic promoter. The term "plant-expressible promoter" includes, but is not restricted to, constitutive, inducible, organ-, tissue-specific or developmentally regulated promoters.

According to the invention, production and/or activity of a polypeptide according to the present invention in a plant or in plant parts is increased by introducing *one or more* polynucleic acids according to the invention into the genome of the plant. More specifically, the constitutive promoter can be, but is not restricted to, one of the following: a 35S promoter (Odell et al. (1985)), a 35S'3 promoter (Hull and Howell

(1987)), the promoter of the nopaline synthase gene ("PNOS") of the Ti-plasmid (Herrera -Estrella, (1983)) or the promoter of the octopine synthase gene ("POCS", De Greve et al. (1982)). It is clear that other constitutive promoters can be used to obtain similar effects. A list of plant-expressible promoters that can be used according to the present invention is given in Table 2.

For specific embodiments of this invention, the use of inducible promoters can provide certain advantages. Modulation of protein levels or protein activity may be required in certain parts of the plant, making it possible to limit modulation to a certain period of culture or developmental stage of the plant.

For specific embodiments of this invention, the use of organ- or tissue-specific or chemical inducible promoters can provide certain advantages. Thus, in specific embodiments of the invention, the gene(s) or part thereof is (are) placed under the control of a promoter directing expression in specific plant tissues or organs, such as for instance roots, leaves, harvestable parts, etc.

It is also possible to use a promoter that can be induced upon the environmental stress conditions. Such promoters can be taken for example from stress-related genes which are regulated directly by an environmental, i.e. preferable abiotic, stress in a plant cell, including genes for which expression is increased, reduced or otherwise altered. These stress related genes comprise genes the expression of which is either induced or repressed by anaerobic stress, flooding stress, cold stress, dehydration stress, drought stress, heat stress or salinity. An exemplary list of such promoters is given in Table 3.

The recombinant polynucleic acids according to the present invention may include further regulatory or other sequences from other genes, such as leader sequences (e.g. the cab22 leader from Petunia), 3' transcription termination and polyadenylation signals (e.g. from the octopine synthase gene or the nopaline synthase gene), plant translation initiation consensus sequences, introns, transcription enhancers and other regulatory elements such as adh intron 1, etc, which is or are operably linked to the gene or a fragment thereof. Additionally, the recombinant polynucleic acid can be constructed and employed to target the gene product of the polynucleic acid of the invention to a specific intracellular compartment within a plant cell on to direct a protein to the extracellular environment. This can generally be obtained by operably joining a DNA sequence encoding a transit or signal peptide to the recombinant polynucleic acid.

The recombinant DNA comprising one or more polynucleic acids according to the present invention may be accompanied by a chimeric marker gene (Hansen et al., 1999 and references therein). The chimeric marker gene can comprise a marker DNA that is operably linked at its 5' end to a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable color to the transformed plant cell, such as the A1 gene (Meyer et al., (1987)), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0 242 246), or can provide antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

According to another embodiment, the present invention invention relates to the use of the polynucleic acids above as selectable marker gene. More preferably, the present invention also relates to the use of the plant DBF2 gene as defined above as selectable marker gene, selection taking place with treatment with a stress condition.

The recombinant DNA vectors according to the present invention comprising the sequences from genes of the invention will typically also comprise a marker gene which confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulforon or Basta.

The present invention also relates to a recombinant host cell transformed with an isolated polynucleic acid as defined above. Said host can be any host known in the art. Preferably said recombinant host cell is a plant cell, yeast, fungi, insect cell, etc. In order to be efficiently expressed in said host, said polynucleic acids can be combined with any promoter known to function in said host system. Methods for transforming said host cells are also well known in the art.

The present invention particularly also relates to a plant cell transformed with at least one recombinant polynucleic acid as defined above.

The present invention also relates to a plant consisting essentially of plant cells transformed with at least one recombinant polynucleic acid as defined above.

A "transgenic plant" refers to a plant comprising a transgene in the genome of essentially all of its cells.

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques (see for example Hansen et al., 1999 for review and WO 99/05902). For example, DNA constructs of the invention may be introduced into the genome of the desired plant host by using techniques such as protoplast transformation, biolistics or microprojectile bombardment or Agrobacterium mediated transformation.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al. (1984).

Electroporation techniques are described in Fromm et al. (1985). Biolistic transformation techniques are described in Klein et al. (1987).

Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional Agrobacterium host vector. The virulence functions of the Agrobacterium host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. Agrobacterium tumefaciens-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch et al. (1984), and Fraley et al. (1983).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium. Plant regeneration from cultured protoplasts is described in Evans et al. (1983); and Binding (1985). Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. (1987).

The polynucleic acids and polypeptides of the invention can be used to confer desired traits on a broad range of plants, including monocotyledonous or dicotyledonous plants, preferably they belong to a plant species of interest in agriculture, wood culture or horticulture, such as a crop plant, root plant, oil producing plant, wood producing plant, fruit producing plant, fodder or forage legume, companion or ornamental or horticultured plant. The plants can include species from the genera Actinidia, Apium, Allium, Ananas, Arachis, Arisaema, Asparagus, Atropa, Avena, Beta,

Brassica, Carica, Cichorium, Citrus, Citrullus, Capsicum, Cucumis, Cucurbita, Cydonia, Daucus, Diospyros, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Ipomoea, Lactuca, Linum, Lolium, Lycopersicon, Malus, Mangifera, Manihot, Majorana, Medicago, Musa, Nicotiana, Oryza, Panicum, Pannesetum, Persea, Petroselinum, Phaseolus, Pisum, Pyrus, Prunus, Raphanus, Rheum, Ribes, Rubus, Saccharum, Secale, Senecio, Sinapis, Solanum, Sorghum, Spinacia, Trigonella, Triticum, Vaccinium, Vitis, Vigna, Zea, and Zingiber. Additional species are not excluded. Crops grown on cultivated lands in arid and semi-arid areas in which irrigation with ground water is needed may advantageously benefit from the invention.

One of skill will recognize that after the recombinant polynucleic acid is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed. As described before, the plant cells, plant tissue, in particular, transgenic plants of the invention display a certain higher or enhanced degree of tolerance (or even resistance) to environmental stress conditions compared to the corresponding wild-type plants. For the meaning of "environmental stress", see supra. In a preferred embodiment of the present invention, the transgenic plant displays increased tolerance to osmotic stress, salt stress, cold and/or heat stress. An increase in tolerance to such environmental stress is understood to refer to a tolerance to a level of such stress which inhibits the growth and productivity of the corresponding untransformed plant, as determined by methodologies known to the art. Such increased tolerance in transgenic plants is related to an increased expression level in the transgenic plant or parts thereof of one ore more of the polynucleic acids of the present invention and/or to an increased level of activity of the polypeptide(s) encoded by said polynucleic acid, as determined by methodologies known to the art. In comparison with their untransformed counterparts, and determined according to methodologies known in the art, a transgenic plant according to the present invention shows an increased growth, viability, metabolism, fertility and/or productivity under mild environmental stress conditions. In the alternative, a transgenic plant according to the invention can grow under environmental stress conditions wherein the untransformed counterparts can not grow. An increase in tolerance to salt stress is understood to refer to the capability of the transgenic plant to grow under stress conditions which inhibit the growth of at least 95% of the parent, non-stress tolerant plants from which the stress tolerant transgenic

plants are derived. Typically, the growth rate of stress tolerant plants of the invention will be inhibited by less than 50%, preferably less than 30%, and most preferably will have a growth rate which is not significantly inhibited by growth conditions which inhibit the growth of at least 95% of the parental, non-stress tolerant plants. In an alternative example, under mild environmental stress conditions, the growth and/or productivity of the transgenic plants is statistically at least 1 % higher than for their untransformed counterparts, preferably more than 5 % higher and most preferably more than 10 % higher.

Any transformed plant obtained according to the invention can be used in a conventional breeding scheme or in *in vitro* plant propagation to produce more transformed plants with the same characteristics and/or can be used to introduce the same characteristic in other varieties of the same or related species.

Furthermore, the characteristic of the transgenic plants of the present invention to maintain normal/rapid/high growth rates under environmental stress conditions can be combined with various approaches to confer environmental stress tolerance with the use of other stress tolerance genes. Some examples of such stress tolerant genes are provided in Holmberg and Bülow (1998). Most prior art approaches which include the introduction of various stress tolerance genes have the drawback that they result in reduced or abnormal growth (compared to non-transgenic controls) under normal, non-stressed conditions, namely stress tolerance comes at the expense of growth and productivity (Kasuga et al., 1999). This correlation between constitutive expression of stress-responsive genes and reduced growth rates under normal growth conditions indicates the presence of cross talk mechanisms between stress response control and growth control.

Furthermore, the characteristic of the transgenic plants of the present invention to display tolerance to environmental stress conditions can be combined with various approaches to confer to plants other stress tolerance genes, e.g., osmotic protectants such as mannitol, proline; glycine-betaine, water-channeling proteins, etc. Thus, the approach of the present invention to confer tolerance to environmental stress conditions to plants can be combined with prior art approaches which include introduction of various stress tolerance genes. Combination of these approaches may have additive and/or synergistic effects in enhancing tolerance or resistance to environmental stress.

Thus, it is immediately evident to the person skilled in the art that the method of the present invention can be employed to produce transgenic stress tolerant plant with

any further desired trait (see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397) comprising:

(i) herbicide tolerance (DE-A 3701623; Stalker (1988)),

(ii) insect resistance (Vaek (1987)),

(iii) virus resistance (Powell (1986), Pappu (1995), Lawson (1996)),

(iv) ozone resistance (Van Camp (1994)),

(v) improving the preserving of fruits (Oeller (1991)),

(vi) improvement of starch composition and/or production (Stark (1992), Visser (1991)),

(vii) altering lipid composition (Voelker (1992)),

(viii) production of (bio)polymers (Poirer (1992)),

(ix) alteration of the flower color, e.g., bu manipulating the anthocyanin and flavonoid biosynthetic pathway (Meyer (1987), WO90/12084),

(x) resistance to bacteria, insects and fungi (Duering (1996), Strittmatter (1995), Estruch (1997)),

(xi) alteration of alkaloid and/or cardia glycoside composition,

(xii) inducing maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO93/25695);

(xiii) higher longevity of the inflorescences/flowers, and

(xvi) stress resistance.

Thus, the present invention relates to any plant cell, plant tissue, or plant which due to genetic engineering displays an enhanced tolerance or resistance to environmental stress obtainable in accordance with the method of the present invention and comprising a further nucleic acid molecule conferring a novel phenotype to the plant such as one of those described above.

The present invention also relates to a callus or calli consisting essentially of plant cells as defined here above. Such transgenic calli can be preferably used for the production of secondary metabolites in plant cell suspension cultures.

The present invention also relates to any other harvestable part, organ or tissue or propagation material of the plant as defined here above.

The present invention also relates to the seed of a transgenic plant as defined here above, comprising said recombinant DNA.

The present invention also relates to the use of any isolated polynucleic acid as defined above to produce transgenic plants.

The present invention also relates to the use of a recombinant polynucleic acid as defined above, to produce transgenic plants, preferably transgenic plants having an enhanced tolerance or resistance to environmental stress conditions. Preferably said polynucleic acid encodes a polypeptide as listed in Table 1.

The present invention also relates to the use of an isolated polynucleic acid as defined above, to produce transgenic callus having an enhanced tolerance or resistance to environmental stress conditions. Preferably said polynucleic acid encodes a polypeptide as listed in Table 1.

The present invention also relates to probes and primers derived from the genes of the invention that are useful for instance for the isolation of additional genes having sequences which are similar to but differ from any of SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, or 121, but which encode a protein having substantially the same biological activity as a protein having the amino acids sequence of any of SEQ ID NO 2 to 120 (even numbers) by techniques known in the art, such as PCR. The presence of a homologous gene in another plant species can for instance be verified by means of Northern of Southern blotting experiments.

The present invention also relates to the cloning of the genomic counterpart of any of the cDNA sequences as represented in SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, or 121. These genomic counterparts can be selected from a genomic library using these cDNA sequences as a probe. The present invention also relates to the coding region as well as the promoter region of any of said genomic clones.

The term "probe" according to the present invention refers to a single-stranded oligonucleotide *sequence* which is designed to specifically hybridize to any of the polynucleic acids of the invention.

The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the nucleic acid strand to be copied. Preferably the primer is about 5-50 nucleotides long. The term "target region" of a probe or a primer according to the present invention is a sequence within the polynucleic acid(s) to which the probe or the primer is completely complementary or partially complementary (i.e. with some degree

of mismatch). It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases.

"Specific hybridization" of a probe to a target region of the polynucleic acid(s) means that the probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions this probe does substantially not form a duplex with other regions of the polynucleic acids present in the sample to be analysed.

"Specific hybridization" of a primer to a target region of the polynucleic acid(s) means that, during the amplification step, said primer forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions the primer does not form a duplex with other regions of the polynucleic acids present in the sample to be analysed. It is to be understood that "duplex" as used hereby, means a duplex that will lead to specific amplification.

Preferably, the probes of the invention are about 5 nucleotides to about 1 Kb long, more preferably from about 10 to 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups which do not essentially alter their hybridization characteristics. The probes according to the present invention preferably include parts of the cDNA sequences of any of the polynucleic acids as defined above.

The present invention also relates to a composition comprising a polynucleic acid sequence as defined above, a polypeptide as defined above, a probe as defined above or a primer as defined above.

The present invention also relates to a pharmaceutical or agrochemical composition comprising said polynucleic acid, a polypeptide of the invention as defined above.

The present invention also relates to antibodies specifically reacting with a protein or polypeptide according to the present invention.

The following Examples describe by way of example the tolerance and/or resistance to several environmental stress conditions observed for transgenic plants and yeast overexpressing some of the polynucleic acids according to the present invention. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) and in volumes 1 and 2 of Ausubel et al. (1994). Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D.

Croy, jointly published by BIOS Scientific Publications Ltd. (UK) and Blackwell Scientific Publications, UK.

These examples and figures are not to be construed as limiting to any of the embodiments of the present invention as set out above. All of the references mentioned herein are incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES AND TABLES

Figure 1. *At-DBF2* encodes a functional homolog of the yeast Dbf2 (A) Comparison of the deduced amino acid sequence of *At-DBF2* with that of yeast *DBF2*. Gaps were introduced to optimize the alignment. Roman numerals above the At-DBF2 sequence indicate the protein kinase catalytic subdomains defined by Hanks et al. (1988). (B) Complementation of *dbf2*. The *dbf2* mutant S7-4A [*MATa dbf2Δ*::URA3 *ura3 leu2 ade5 trp1 his7*] (Toyn and Johnston, 1994) (B1) forms swollen pairs of daughter cells (dumbbells) at restrictive temperature (37° C). The defective morphology of the *dbf2* mutant can be complemented by transformation with the pYX112 centromeric plasmid (Ingenius, R&D system) containing the *At-DBF2* cDNA (B2) or *DBF2* (B3); wild type (CG378 strain, *MATa ade5 leu2 trp1 ura3*) (B4). Log phase cultures were shifted from 28°C to 37°C and photographed after 16 hours. After 16 hours, 98% of the S7-4A cells arrested with a dumbbell morphology (B1) whereas 6,1 and 0% of dumbbells were observed in B1, B3 and B4. Strains were kindly provided by (Dr Lindl, Max Planck Institut fur Zuchtungsforschung, Koln, Germany).

Figure 2. Overexpression of *DBF2* or *At-DBF2* enhances tolerance to osmotic, salt, heat and cold stress. Yeast cells were grown in YPD and cell density was adjusted to OD600 at 2. (1) DY, (2) DY transformed with pYX212 containing *DBF2*, pYX-YDBF2 , (3) DY transformed with vector alone or (4) with vector containing *At-DBF2*, pYX-AtDBF2. Serial dilutions were made in step1:10. Ten µl of each dilution was spotted on solid YPD medium (control) supplemented with 2M sorbitol (osmotic stress) or 1.2 M NaCl (salt stress) or 4µl H_2O_2 (oxidative stress) and incubated at 28°C or at 42°C (heat stress) or at 4°C (cold stress) for 3 days.

Figure 3. *DBF2* and *At-DBF2* are induced by stress. (a) Northern analysis showing the kinetics of *At-DBF2* induction in plants treated with PEG 6000 20 % and the one of *DBF2* in yeast treated with sorbitol 2M for the time indicated. (b) Northern analysis *of At-DBF2* in 10 day-old-plants grown for 5 hours in control conditions (as described in Verbruggen et al. 1993) (1), at 37°C (2), with PEG 6000 20 % (3), NaCl 1% (4), at 4°C (5) or with 0.4 mM H_2O_2 (6); and of *DBF2* in yeast cells grown for 11/2 hour in YPD (1), at 37°C (2), with sorbitol 2M (3), with NaCl 1.2 M (4), at 4°C (5) or with 0.4 mM H_2O_2 (6). Control of loading has been done with EtBr staining and is shown under each Northern analysis.

(c) Western analysis of At-DBF2 in Arabidopsis. Samples are similar to those analysed in (b). Antibodies used were raised against yeast Dbf2 and kindly provided by Dr L. Leindl (Max Planck Institut fur Zuchtungsforschung, Koln, Germany).

Figure 4. *DBF2* overexpression can suppress *hog1* osmosensitivity. The *hog1* mutant (4) [W303-1A, *MATa*, *hog1Δ:: TRP1*] and wild type (W303) (1) were kindly provided by Dr Thevelein (Katholieke Universiteit Leuven, Belgium). The *hog1* mutant was transformed with pYX-YDBF2 (2) or pYX-AtDBF2 (3). Each of the 4 strains was grown for 16 hours in YPD (rich medium), and cell density was adjusted to OD600 at 2. Serial dilutions, 1:10 were made at five consecutive steps. Ten microliter of each dilution was spotted on solid YPD medium (control) or solid YPD medium supplemented with 0,9 M NaCl and incubated at 28°C for 3 days.

Figure 5. *T-DBF2* (*Nicotiana tabacum* DBF2) is periodically expressed during plant cell cycle. Tobacco *DBF2* expression has been followed in BY2 cells synchronised with aphidicolin (a & b) or with propyzamide (c & d) with *At-DBF2* as probe. The measure of relative rate of DNA synthesis and of the mitotic index, the use of the cell cycle markers *CYCB1.2* and *H4* markers have been previously described (Reicheld et al., 1995). *T-DBF2* transcript levels were quantified from the blots shown in b and d using a PhosphorImager (Molecular Dynamics).

Figure 6. shows the results of a comparison of the growth of *A. thaliana* plants transformed with the following constructs: P35S-At-DBF2 (upper left and bottom right section), P35S control (upper right section) and P35S-antisense At-DBF2 (bottom left section) upon applying a salt stress of 200 mM NaCl overnight.

Figure 7 shows the results of a comparison of the growth of *A. thaliana* plants transformed with the following constructs: P35S-At-DBF2 (upper left and bottom right section), P35S control (upper right section) and P35S-antisense At-DBF2 (bottom left section) upon applying an osmotic stress induced by 20% PEG overnight.

Figure 8 shows the results of a comparison of the growth of *A. thaliana* plants transformed with the following constructs: P35S-At-DBF2 (upper left and bottom right section), P35S control (upper right section) and P35S-antisense At-DBF2 (bottom left

section) upon applying a cold stress by gradually decreasing the temperature untill – 7°C.

Figure 9 shows the results of a comparison of the growth of *A. thaliana* plants transformed with the following constructs: P35S-At-DBF2 (upper left and bottom right section), P35S control (upper right section) and P35S-antisense At-DBF2 (bottom left section) upon applying a heat stress of 2 hours at 48°C.

Figure 10 shows the results of a comparison of the growth of *A. thaliana* plants transformed with the following constructs: P35S-At-DBF2 (upper left and bottom right section), P35S control (upper right section) and P35S-antisense At-DBF2 (bottom left section). It can be concluded that the P35S-At-DBF2 transformed plants do not show morphological abnormalities compared to the control transgenic plants.

Figure 11 shows the results of a salt stress tolerance test with transgenic *A. thaliana* plants overexpressing HSP 17.6A (A) or c74 (B). The control plants (bottom left in A en B) is a transgenic line tranformed with pBIN-35S-CaMVter. The other sections in A are 5 independently obtained transgenic lines overexpressing HSP17.6A. The other sections in B are 5 independently obtained transgenic lines overexpressing c74.

Figure 12 shows the influence of *At-DBF2* expression in sense and antisense orientations on stress tolerance. BY2 cells were transformed by *A. tumefaciens* with recombinant T-DNA vectors containing At-DBF2 driven by CaMV 35S RNA promoter, pBIN-35S-At-DBF2 (upper left and right sections in A or diamonds in B), the CaMV 35S promoter and terminator pBIN-35S-CaMVter (bottom left sections in A or triangles in B), or antisense *At-DBF2* under the control of the CaMV 35S promoter pBIN-35S-ASAt-DBF2 (bottom right sections in A or circles in B). (A) Picture of the same amounts of transgenic cells after 3 weeks of growth on solid medium supplemented with 300 mM NaCl, 25% PEG, 2mM H_2O_2, or at 47°C (heat). (B) Growth of suspension cells in liquid medium. Upon stress, growth was measured as fresh weigth and expressed as a percentage of unstressed growth (control) (a). Stresses were applied after subculturing (= day 0) at indicated temperatures (e) and concentrations of NaCl (b) PEG (c), and H_2O_2 (f). For the cold shock (d), cells were maintained at 0°C for 2 days before the 2-week culture at 22°C. For each construction data of three

independent transgenic lines were pooled. To not overload the figure, SDs are not shown (maximum 15% of measured values). (C) Northern analysis of At-DBF2+TDBF2, kin1, and HSP17.6. Total RNAs were extracted from independent lines transformed with pBIN-35S-At-DBF2 (1) and (2), pBIN-35S-CaMter (3), and pBIN-35S-ASAt-DBF2 (4). Osmotic stress was induced with 10% PEG treatment for 5 hr (stressed).

Figure 13 shows the results of the growth of *A. thaliana* plants transformed with p35S-AtHSP17.6A and P35S control (upper right section) upon applying an osmotic stress induced by 20% PEG overnight. The results of two independent experiments are shown, each performed with 3 independently obtained transgenic lines overexpressing At-HSP17.6A (upper left and bottom left and right).

Figure 14 shows the results of the germination of *A. thaliana* plants transformed with p35S-Atc74 and P35S control (bottom section) on mineral medium supplemented with 125 mM NaCl. The results of two independent experiments are shown, each performed with 2 independently obtained transgenic lines overexpressing Atc74 (2 upper sections).

Table 1. Classification of the *Arabidopsis thaliana* clones isolated in Example 2. Clones isolated according to the description in example 2 have been analyzed on their potential to confer tolerance.According to the method described in example 2, the tolerance of different yeast transformants expressing an Arabidopsis cDNA to osmotic stress and salt stress was compared with the tolerance of DY wild type cells.

+	:	similar growth to the DY wild type cells;
++	:	growth of the transformant is visible at a 10-fold higher dilution (1:10) than control (1:1);
+++	:	growth of the transformant is visible at a 100-fold higher dilution (1:100) than control (1:1);
++++	:	growth of the transformant is visible at a 1000-fold higher dilution (1:1000) than control (1:1).

Table 2. Exemplary plant-expressible promoters for use in the performance of the present invention.

Table 3. Exemplary stress-inducible promoters for use in the performance of the present invention.

EXAMPLES

Example 1. Construction of the cDNA library.

Total RNA has been isolated from green siliques from *Arabidopsis thaliana* by grinding 1 g of siliques in 4 ml extraction buffer (100 mM tris-Hcl, pH 8, 10 mM EDTA, 100 mM LiCl) at 4° C, followed by phenolisation and chloroform: isoamylalcohol (24:1) extraction. To the aqueous phase, LiCl was added up to a final concentration of 2M, and the total RNA was allowed to precipitate overnight at 4°C. After centrifugation, the pellet was redissolved in 400 µl H_2O and reprecipitated with ethanol. Poly(A) messenger RNA was isolated from the total RNA by binding it to an oligo-dT cellulose spun column (Pharmacia), washing the column three times with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA , 0.5 M NaCl and eluting the mRNA with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 65° C.

The eluate was precipitated with ethanol, and cDNA was synthesized using MMLV- reverse transcriptase (Pharmacia) and a $d(T)_{14}$-XhoI primer for the first strand and *E. coli* DNA polymerase I (Pharmacia) for the second strand.

Example 2. Yeast transformation and selection for osmotolerance.

The cDNA was cloned into pYX vectors (Ingenius, R&D systems; 2 µ based pYX 212 for bank 1, ARS/CEN based pYX112 for bank 2) as EcoRI - XhoI fragments, using an Eco RI/Not I adaptor.

In these constructs, the cDNA is under the control of the strong constitutive TPI promoter. The yeast strain DY (MATa, *his3, can1-100, ade2, leu2, trp1, ura3*::3xSV40AP1-lacZ; kindly provided by N. Jones, Imperial Cancer Research Fund, London, UK) has been transformed with these cDNA libraries, using the Lithium Acetate transformation procedure (Gietz and Schietsl, 1995). After transformation with the Arabidopsis cDNA bank, transformants have been selected for the ability to grow in the presence of 100mM LiCl in a stepwise selection (Lee et al., 1999). LiCl is commonly used for salt tolerance screening in yeast (Haro et al. 1991). Several *A. thaliana* genes, conferring osmotolerance to the yeast, have been isolated (Table 1). To further analyse the potential of the selected Arabidopsis cDNA's to confer tolerance to environmental stress in yeast, each yeast transformant expressing such selected Arabidopsis cDNA's has been exposed to osmotic stress and salt stress. Each of the transformants was therefore grown for 16 hours in YPD (rich medium), and cell density was adjusted to OD_{600} at 2. Serial dilutions, 1:10, were made at three consecutive

steps. Ten microliters of each dilution was spotted on solid YPD medium (control) supplemented with 2 M sorbitol (osmotis stress) or 1.2 M NaCl (salt stress) and incubated at 28°C for 3 days. The results of this drop growth test (see also Lee et al., 1999) are shown in Table 1.

Example 3. Characterization of *At-DBF2*.

At-DBF2, a 1.8 kb cDNA (SEQ ID NO 1) has been identified in this screening that encodes a predicted 60.2 kDa protein showing 81 % similarity with the yeast Dbf2 transcriptional regulator. Homology (less than 40% similarity) has also be found with the putative Dbf2 homologues in human, C. elegans and Drosophila (named Ndr for nulear Dbf2 related, Millward *et al.* 1995). The *At-DBF2* deduced protein sequence (SEQ ID NO 2) contains the 11 domains of protein kinases (Figure 1A). Amino acids lying between the invariant residues D and N of domain VI do not match the features of serine/threonine specificity (LKPE) defined by Hanks *et al.* (1988) but the GSPDYIALE peptide in domain VIII does well indicate serine/threonine specificity and *At-DBF2* can complement the yeast *dbf2* mutant (Figure 1B).

In mature Arabidopsis plants, *At-DBF2* is expressed in all tested organs. The highest abundance of transcripts has been found in siliques. A Southern analysis in Arabidopsis, tobacco and tomato has revealed that *DBF2* seems to be conserved in plants (see Example 13 below). As *At-DBF2* has been identified in a screening for LiCl tolerance, its effect in other stress situations has been tested in yeast (Figure 2).

Example 4. Overexpression of *Arabidopsis* and *Saccharomyces cerevisiae DBF2* enhances cold, heat, salt and drought tolerance in yeast.

In order to test whether the effect was specific to the plant gene, the yeast *DBF2* gene has been overexpressed in the same vector. Upon a drop growth test (Figure 2 and Lee et al., 1999). A remarkable enhancement of stress tolerance can be seen at 42°C, during osmotic stress (sorbitol), and after salt and cold treatments in yeast. There is no difference between stress tolerance afforded by the plant or the yeast gene. The enhancement of stress tolerance due to the overexpression of *At-DBF2* or *DBF2* reflects a role for these genes in stress situations. Therefore yeast and Arabidopsis plants have been exposed to sorbitol- and PEG-induced osmotic stress. *At-DBF2* as well as *DBF2* is induced rapidly (1 to 2 hours) and transiently upon osmotic stress (Figure 3A). The expression of *At-DBF2* and *DBF2* has been analyzed during other environmental stresses in Arabidopsis plants or in yeast cells after the

time corresponding to the highest induction seen in Fig. 3A (Figure 3B). In plant as in yeast, there is a clear induction after heat, salt, osmotic and to a lesser extent after cold, which perfectly correlates with stresses to which the overexpression enhances tolerance. However, many genes are induced upon stress without relevant adaptive role, amongst others because post-transcriptional mechanisms inhibit subsequent translation. Here *At-DBF2* protein amount, as detected by anti-Dbf2 antibodies, clearly increased upon stress (Figure 3C).

Example 5. Both *At-DBF2* and *DBF2* can functionally complement the *hog1* mutation.

To investigate a possible interaction between stress signaling pathways and *DBF2*, the salt sensitive hog1 mutant was transformed with *At-BDF2* and *DBF2*. The *HOG1* MAP kinase pathway regulates osmotic induction of transcription in yeast (Schuller *et al.* 1994). The osmosensitivity of the mutant could be recovered by the overexpression of both *DBF2* and *At-DBF2* (Figure 4).

Example 6. *At-DBF2* is cell cycle regulated.

DBF2 expression is cell cycle regulated where it plays a role in DNA synthesis initiation but also in nuclear division through its association with the CCR4 complex (Komarnitsky *et al.* 1998, Johnston *et al.* 1990). This regulation was investigated in plants. A tobacco BY-2 cell line in which the highest level of culture synchronization, compared with other plant cell lines has been achieved so far (Shaul *et al.* 1996, Reicheld *et al.* 1995) was used. Stationary phase cells were diluted into fresh medium and treated with aphidicolin (blocking cells in the beginning of the S phase) for 24 hours, then washed. The percentage of synchronous mitosis after release from the aphidicolin block was about 65 % (Figure 5A-B). A 1.6-Kb tobacco *DBF2* homologue (*T-DBF2*) could be detected on Northern blot with the *At-DBF2* as a probe. *T-DBF2* steady-state transcript level clearly oscillates during the cell cycle and is mainly present during S, decreases during G2 until late M from where it increases until a peak in S phase. *T-DBF2* expression occurs clearly before *CYCB1.2* (a marker of G2-M phases), but parallels the one of H4 (a S phase marker) except at the S/G2 transition, where *T-DBF2* transcripts decline earlier, and at the M/G1 transition, where *T-DBF2* expression increases earlier. The use of the cell cycle markers CYCB1.2 and H4 is described in Reicheld et al.

To follow unperturbed G1 and S phases, BY2 cell suspension was synchronized using a double blocking procedure (Nagata *et al.*,1992). After the release from the aphidicoline block, cells are treated for 4 hours with propyzamide in the beginning of the preprophase. The percentage of synchronous mitosis after the release from the propyzamide block was higher than 75%. *T-DBF2* was periodically expressed with an undetectable expression until late M, a sharp increase in G1 and a peak in mid S (Figure 5C-D) which confirms results of Figures 5A-B. However a function for the plant *DBF2* in cell cycle can only be assigned with measurement of the kinase activity. In yeast, *DBF2* transcript levels do not correlate with kinase activation which occurs by dephosphorylation (Toyn and Johnson, 1994). The precise function of Dbf2 in regulation of the cell cycle is not known. An essential role has been proposed during anaphase or telophase. No activity has been measured in G1 despite evidence for a role for Dbf2 in initiation of DNA synthesis.

As other proteins recently identified, Dbf2 controls the M/G1 transition which is a major cell cycle transition in yeast (Aerne *et al.* 1998). The existence of a M/G1 control checkpoint has been suggested in plant cells (Hemmerlin and Bach 1998) but its importance compared to G1/S and G2/M has not been investigated.

Overexpression of *DBF2* in yeast results in kinase activity throughout the cell cycle, which may be due to the saturation of a post-translational deactivating mechanism (Toyn and Johnston, 1994). Overexpression of the functionnally conserved *At-DBF2* has most probably the same effect. However, the presence of Dbf2 kinase activity at the wrong time in the cell cycle does apparently not affect its progression. In marked contrast constitutive activity has a marked effect on stress tolerance. The role played by *At-DBF2* or *DBF2* in stress is most probably independent from the cell division cycle. *At-DBF2* expression is present in all plant organs (abundant expression is observed in stems where only 1-2 % cells have a mitotic activity) and can be rapidly induced upon stress. However, a link with the cell cycle is not excluded. Higher stress tolerance in yeast overexpressing *DBF2* or *At-DBF2* may be correlated to the overproduction of the kinase in G1 where yeast cells are particularly sensitive to stress. Most plant cells are also thought to be blocked in G1 but the relationship with stress response is poorly known.

Example 7. **Tobacco cell transformation and rec mbinant T-DNA Vector construction**

BY2 cells were stably transformed as described (Shaul et al., 1996) by *Agrobacterium tumefaciens* C58C1Rif^R (pGV2260) strain (Deblaere et al., 1985) carrying pBIN-35S-At-DBF2 or pBIN-35S-ASAt-DBF2 recombinant binary vectors. PBIN-35S-At-DBF2 is the plant binary vector pBIN m-gfp4 in which the BamHI-SacI fragment containing the gfp reporter gene was replaced with a BamHI-SacI fragment containing the At-DBF2 cDNA from pYX-At-DBF2. p-Bin-35S-CaMVter is the plant binary vector pBIN19 in the HindIII-SacI restriction sites of which the hindIII-SacI fragment of pDH51 containing the cauliflower mosaic virus (CaMV) 35S RNA promoter and terminator was cloned. pBIN-35S-ASAt-DBF2 is the pBIN-35S-CaMVter vector in which the At-DBF2 cDNA was cloned in the antisense orientation from pYXAt-DBF2 in the BamHI-SmaI restriction sites, between the CaMV 35S RNA promoter and terminator. More details are described in Lee et al. (1999).

Example 8. Overexpression of At-DBF2 sense and antisense RNA in plant cells

Transgenic plant cells overexpressing *At-DBF2* were generated to test the role of this protein in stress tolerance *in planta*. Tobacco BY2 cells were stably transformed by *A. tumefaciens* carrying the *At-DBF2* cDNA driven by the strong constitutive CaMV 35S RNA promoter. The antisense *At-DBF2* RNA also was overexpressed under the control of the same promoter. Control lines were obtained by transforming tobacco BY2 cells with pBIN-35S-CaMVter. Three independently obtained *At-DBF2*-overexpressing tobacco transgenic cell lines have been selected with a high and similar *At-DBF2* expression and analysed further. Three tobacco transgenic cell lines overexpressing antisense *At-DBF2* were chosen that showed an undetectable tobacco DBF2 transcript level. Both the overexpression of *At-DBF2* and the down-regulation of the endogenous gene by the antisense strategy did not result in significant differences in growth after 2 weeks (Fig. 12A and 12B). On the contrary, marked differences in growth were observed after a 2-week treatment with NaCl, PEG-induced drought, cold, or high temperatures. Transgenic lines that overexpressed *At-DBF2* were clearly more tolerant than control lines. Inhibition of the endogenous DBF2 expression was correlated with a higher sensitivity to those stresses. To understand the basis of stress

tolerance in *At-DBF2*-overexpressing plant cells, expression of stress-induced genes was followed in control and stress conditions (Fig. 12C). Tobacco kin1 and HSP17.6A homologues already were induced in *At-DBF2*-overexpressing tobacco cells in control conditions to a level similar to that observed during stress conditions (PEG-induced drought), suggesting that *At-DBF2* overexpression may mimic a stress signal.

Example 9. Arabidopsis transformation and recombinant T-DNA vector construction with genes conferring tolerance to environmental stress

Arabidopsis were stably transformed as described in Clarke, Wei and Lindsey (1992) by Agrobacterium tumefaciens C58C1RifR (pGV2260) strains carrying pBIN-35S-At-DBF2, pBIN-35S-At-HSP17.6A, pBIN-35S-At-c74 recombinant binary vectors. pBIN-35S-At-DBF2 is described in Lee et al. 1999. pBIN-35S-At-HSP17.6A recombinant binary vector was constructed as following: the EcoRI-XhoI fragment containing At-HSP17.6A cDNA in pYX-HSP17.6A (recombinant pYX212) was first cloned in pYES2 (Invitrogen) resulting in pYES-HSP17.6A. Than the BamHI-SphI fragment of pYES-HSP17.6A containing the At-HSP17.6A cDNA was cloned in the plant binary vector pBIN m-gfp4 in which the BamHI-SacI fragment containing the gfp receptor gene was deleted and replaced by the At-HSP17.6A cDNA. The 3' protruding ends generated by SacI and SphI were blunt ended by T4 DNA polymerase. pBIN-35S-c74 was constructed with a similar strategy as pBIN-35S-AtHSP17.6A with an intermediary pYES-Atc74 vector. The At-c74 cDNA was first amplified with PCR using the primers 5' AAA AAA CAC ATA CAG GAA TTC 3' (SEQ ID NO 122) and 5' AGT TAG CTA GCT GAG CTC GAG 3' (SEQ ID NO 123), then cloned "blunt ended" in the vector pYES2 cut with NotI and BstXI and blunt ended with T4 DNA polymerase. Subsequently, the BamHI-SphI fragment of pYES-c74 was cloned in pBINm-gfp4 as explained supra.

Example 10. Tolerance to environmental stress in plant cells

Transgenic calli were isolated from each of the transgenic Arabidopsis lines transformed with At-DBF2, At-HSP17.6A and At-c74. The growth of these transgenic calli during salt stress was measured and compared with control calli derived from transgenic Arabidopsis lines transformed with pBIN-35S-CaMVter. Callus pieces (25 for each transgenic line) of similar fresh weight (50 to 100 mg) were therefor grown on callus inducing medium (Clarke et al., 1992) supplemented with 200mM NaCl. After two weeks, from visual inspection, it was clear that transgenic calli transformed with

At-DBF2 or At-HSP17.6A or At-c74 looked much better than control transgenic calli transformed with pBIN-35S-CaMVter. The latter calli turned yellow and started dying. To confirm the observation, the fresh weight of the calli was measured. In comparison with the control transgenic calli, the fresh weight of the transgenic calli was for each of the three lines at least five times higher than the fresh weight of the control trangenic calli.

Example 11. Tolerance to environmental stress in plants.

Seeds from transgenic Arabidopsis plants tranformed with pBIN-35S-At-DBF2, p-BIN-35S-At-c74, or pBIN-35S-At-HSP17.6A, were sown in bulk on nylon filters (as described in Verbruggen et al. 1993) placed on solid K1 medium supplemented with kanamycin (75 micrograms/ml). For each recombinant pBIN binary vector at least five independent transgenic lines were tested for stress tolerance. In each of these lines overexpression of the transgene has been confirmed with Northern hybridisation experiments. Control plants were the ones transformed with pBIN-35S-CaMVter and transgenic plants transformed with pBIN-35S-AS+At-DBF2. After sowing, seeds were kept overnight at 4 degrees (to enhance germination). Growth was at 22 degrees, 60 % humidity, 16 hours light/8 hours dark, 70 microeinsteins. After 9 days growth, filters were transferred to liquid K1 medium supplemented with 200 mM NaCl for overnight incubation. Plants were allowed to recover for 5 to 6 days by transferring the filters to solid K1 medium. Under these conditions, the control transgenic plants turned yellow, their growth was inhibited and eventually they died. On the contrary, the transgenic lines transformed with At-DBF2 or At-HSP17.6A or At-c74 survived very well (Figure 6 and Figure 11).

To further evaluate the scope of protection to environmental stress, transgenic plants were exposed to osmotic stress. Therefor seeds from transgenic Arabidopsis plants transformed with pBIN-35S-At-DBF2, pBIN-35S-At-c74 or pBIN-35S-At-HSP17.6A were sown in bulk on nylon filters (as described in Verbruggen et al. 1993) placed on solid K1 medium supplemented with kanamycin (75 micrograms/ml). For each recombinant pBIN binary vector at least five independent transgenic lines were tested for stress tolerance. In each of these lines overexpression of the transgene has been confirmed with Northern hybridisation experiments. Control plants were the ones transformed with pBIN-35S-CaMVter and transgenic plants transformed with pBIN-35S-ASAt-DBF2. After sowing, seeds were kept overnight at 4 degrees (to enhance germination). Growth was at 22 degrees, 60 % humidity, 16 hours light/8 hours dark,

70 microeinsteins. After 9 days growth, filters were transferred to liquid K1 medium supplemented with 20 % polyethylene glycol for overnight incubation. Plants were allowed to recover for 5 to 6 days by transferring the filters to solid K1 medium. Under these conditions, the control transgenic plants turned yellow, their growth was inhibited and eventually they died. On the contrary, the transgenic lines transformed with At-DBF2, At-HSP17.6A or At-c74 survived very well (see Figure 7 and 13). Their growth was comparable to growth on control medium without polyethylene glycol.

To further analyse the scope of protection to environmental stress, transgenic plants were exposed to high and low temperatures. Therefor seeds from transgenic plants transformed with pBIN-35S-At-DBF2 or pBIN-35S-At-c74 were sown in bulk on nylon filters (as described in Verbruggen et al. 1993) placed on solid K1 medium supplemented with kanamycin (75 micrograms/ml). For each recombinant pBIN binary vector at least five independent transgenic lines were tested for stress tolerance. In each of these lines overexpression of the transgene has been confirmed with Northern hybridisation experiments. Control plants were the ones transformed with pBIN-35S-CaMVter and transgenic plants transformed with pBIN-35S-ASAt-DBF2. After sowing, seeds were kept overnight at 4 degrees (to enhance germination). Growth was at 22 degrees, 60 % humidity, 16 hours light/8 hours dark, 70 microeinsteins. After 9 days growth, for the experiments with high temperature stress, plants were exposed to 48°C for two hours. For the experiments with low temperature stress, plants were exposed to gradually decreasing temperatures, down to -7°C. Plants were allowed to recover for 5 to 6 days by transferring the filters to solid K1 medium.

Under both low temperature and high temperature stress, the growth of control transgenic plants was inhibited and eventually they died. The transgenic lines transformed with At-DBF2 or At-c74 survived very well. Their growth was comparable to growth under control conditions with normal temperature (see Figure 8 and 9).

To further analyse the scope of protection to environmental stress, transgenic plants were exposed to salt stress during germination. Sterilized mature seeds from transgenic plants transformed with pBIN-35S-At-DBF2 or pBIN-35S-At-c74 were placed on top of petri dishes containing MS (Murashige and Skoog) medium with 0,8 % agar and 30 g l^{-1} sucrose. Control plants were the ones transformed with pBIN-35S-CaMVter. Prior to germination and pH 5.7 adjustment, NaCl was added to a final concentration of 125 mM. Three petri dishes with a mean of 40-50 seeds per dish were used per treatment in every experiment. The complete experiment was repeated

twice. Seed germination at 22°C was followed. Seeds were considered to germinate after radical and green cotyledon emergency occurred.

On control medium (without 125 mM NaCl), germination of all transgenic lines was very similar to each other and to wild type plants. On medium supplemented with 125 mM NaCl, seeds from transgenic lines overexpressing At-DBF2 or At-c74 germinate significantly better than control transgenic lines. Less than 10 % of the seeds from transgenic lines transformed with pBIN-35S-CaMVter germinate under these conditions. In contrast, more than 70 % of the seeds from trangenic lines overexpressing At-DBF2 or At-c74 germinate on medium containing 125 mM NaCl (Figure 14).

Example 12. Southern hybridisation of At-DBF2 genes in other plants

To investigate whether *DBF2* homologues exist in other plant species, a Southern hybridisation analysis was performed using the full length *At-DBF2* as a probe. Genomic DNA was extracted from tobacco, tomato and rice according to Dellaporta et al. (1983) and further purified by phenol :chloroform extractions.

DNA (10 µg) was digested with restriction enzymes and separated on 1% (w/v) agarose gels using Lambda DNA digested with Hind III as molecular size standards. The DNA was transformed on to nylon membranes (Hybond N ; Amersham, little Chalfont, UK) in 0.4 N NaOH. Filters were UV-cross-linked for 30 seconds, prehybridized for 3 hours at 56°C in hybridization solution (2x SSPE, 0.1%(w/v) SDS, 5x Denhardt solution) using 200 gm^{-3} denatured salmon sperm DNA, and hybridized overnight with radiolabelled probes. 1X SSPE was 0.15 M NaCl/ 0.01 M sodium dihydrogen phosphate/ 1 mM EDTA

Filters were washed at 56°C in 2x SSPE, 0.1% (w/v) SDS for 20 min, then 1x SSPE, 0.1% (w/v) SDS for 20 min, and finally in 0.1x SSPE, 0.1% (w/v) SDS for 20 min. Filters were exposed to X-ray film (Kodak X-AR ; Kodak, NY, USA) in the presence of intensifying screens for 24 hours.

The results of the hybridisation experiments show that tobacco, tomato and rice have at least one homologue to At-DBF2.

Tabel 1

putative function in	Features of encoded protein	SEQ ID NO.	Growth on medium with1,2 M NaCl	growth on medium with 2,0 M sorbitol
signalling	Similar to a yeast DBF2 cell cycle protein	1	++++	++++
metabolism	HSP17.6A	3	++++	++++
unknown	C74	5	+++	+++
metabolism	Similar to ADH2	7	+	++++
metabolism	Similar to D. melanogaster catalase/catalase 3	9	++++	+
metabolism	Similar to the HSP90 heat shock protein family	11	++++	++++
metabolism	similar to phosphoenolpyruvate carboxylase	13	+	+++
metabolism	pathogen related proteins, class 10	15	+	++++
metabolism	Arabidopsis ascorbate peroxidase	17	++++	++++
metabolism	similar to phosphatase binding protein	19	++++	++++
metabolism	similar to phosphatase binding protein	21	++++	++++
metabolism	similar to retinol dehydrogenase	23	+++	++++
metabolism	similar to retinol dehydrogenase	25	++++	++++
metabolism	ribosomal protein	27	++++	++++
metabolism	ribosomal protein	29	++++	++++
metabolism	similar to a protein transporter (kinase homolog)	31	++++	++++
metabolism	similar to a peptide transporter	33	++++	+
metabolism	similar to a wheat low affinity cation transporter LCT1	35	++++	++++
metabolism	similar to yeast iso-1-cytochrome c (CYC-1)	37	++++	++++
metabolism	similar to yeast OSM1	39	++++	++++
metabolism	similar to yeast copper uptake gene (CUP1)	41	++++	+++
metabolism	similar to yeast UV-induced damage repair protein (RAD7)	43	++++	++++
metabolism	electron transporter, apocytochrome b	45	++++	++++
metabolism	similar to membrane lipoprotein LPPL1	47	++++	++++
metabolism	similar to tobacco auxin binding protein	49	+	++++
metabolism	similar to tobacco cytokinin binding protein CBP 57	51	+++	++++
signalling	similar to calcium binding protein yeast calcineurin B	53	+++	++++

signalling	similar to calcium binding protein glycine max calnexin	55	++++	+++
signalling	similar to calcium binding protein Dictyostelium discoideum calreticulin	57	++++	++++
signalling	similar to calcium binding protein calmodulin 1	59	++++	+
signalling	similar to calcium binding protein calmodulin 2	61	+	++++
signalling	MAP kinase kinase, homologous to Dyctyostelium mekA (DdMek1)	63	++++	+++
signalling	similar to human adenosine kinase	65	+	++++
signalling	similar to human tyrosine kinase	67	++++	++++
signalling	similar to common ice plant tyrosine kinase	69	++++	++++
signalling	similar to the yeast protein kinase C receptor	71	++++	++++
signalling	similar to tobacco and Arabidopsis HAT7 homeotic protein	73	++	++++
signalling	similar to E. coli sigma factor regulator (RSEB)	75	+	++++
signalling	similar to human protein phosphatase 2C	77	++++	++++
metabolism	late embryogenesis abundant proteins, Arabidopsis LEA protein 10 & 14	79	++	++++
metabolism	late embryogenesis abundant proteins, Arabidopsis LEA protein 10 & 14	81	++	++++
metabolism	pathogen related proteins, class 10	83	++++	++++
metabolism	cell wall peroxidase	85	++++	+++
metabolism	ribosomal protein	87	+++	++++
metabolism	salt stress induced protein, SAS 1	89	++++	++++
metabolism	PR gene (AIG2)	91	++++	++++
metabolism	MT1c	93	++++	++++
metabolism	IPP2 (Isopentenyl diphosphate)	95	+++	++++
metabolism	chlorophyll a/b binding protein	97	+++	+++
metabolism	glutathione transferase	99	++	++++
signalling	cold- and ABA inducible, calcium dependent – kinase, Kin1	101	++++	++++
signalling	MAP kinase, Atmpk1	103	++	++++
signalling	Arabidopsis cell cycle protein histone H2A	105	++++	++++
unknown	chromosome 4 – sequence	107	+++	++++
unknown	chromosome 4 – sequence	109	+	++++
unknown	chromosome 5 – sequence	111	++++	+++
unknown	chromosome 5 – sequence	113	++++	++
unknown	chromosome 5 – sequence	115	++++	++++
unknown	chromosome 5 – sequence	117	+	++++
unknown	chromosome 5 – sequence	119	+	++++

signalling	similar to calcium binding protein centrin (caltractin)	121	++++	++++

TABLE 2
EXEMPLARY PLANT-EXPRESSIBLE PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
α-amylase (*Amy32b*)	Aleurone	Lanahan *et al* (1992); Skriver *et al.* (1991)
cathepsin β-like gene	Aleurone	Cejudo *et al.* (1992)
Agrobacterium rhizogenes rolB	Cambium	Nilsson *et al.* (1997)
PRP genes	cell wall	http://salus.medium.edu/mmg/tierney/html
barley *ltr1* promoter	Endosperm	
synthetic promoter	Endosperm	Vicente-Carbajosa *et al.*(1998)
AtPRP4	Flowers	http://salus.medium.edu/mmg/tierney/html
chalene synthase (chsA)	Flowers	van der Meer *et al.* (1990)
apetala-3	Flowers	
Chitinase	fruit (berries, grapes, etc)	Thomas *et al.* CSIRO Plant Industry, Urrbrae, South Australia, Australia; http://winetitles.com.au/gwrdc/csh95-1.html
rbcs-3A	green tissue (eg leaf)	Lam *et al.* (1990); Tucker *et al.* (1992)
leaf-specific genes	Leaf	Baszczynski *et al.* (1988)
AtPRP4	Leaf	http://salus.medium.edu/mmg/tierney/html
Pinus cab-6	Leaf	Yamamoto *et al.* (1994)
SAM22	Senescent leaf	Crowell *et al.* (1992)
R. japonicum nif gene	Nodule	United States Patent No. 4, 803, 165
B. japonicum nifH gene	Nodule	United States Patent No. 5, 008, 194

GmENOD40	Nodule	Yang *et al.* (1993)
PEP carboxylase (PEPC)	Nodule	Pathirana *et al.* (1992)
Leghaemoglobin (Lb)	Nodule	Gordon *et al.* (1993)
Tungro bacilliform virus gene	Phloem	Bhattacharyya-Pakrasi *et al.* (1992)
sucrose-binding protein gene	plasma membrane	Grimes *et al.* (1992)
pollen-specific genes	pollen; microspore	Albani *et al.* (1990); Albani *et al.* (1991)
maize pollen-specific gene	Pollen	Hamilton *et al.* (1992)
sunflower pollen-expressed gene	Pollen	Baltz *et al.* (1992)
B. napus pollen-specific gene	pollen;anther; tapetum	Arnoldo *et al.* (1992)
root-expressible genes	Roots	Tingey *et al.* (1987); An *et al.* (1988);
tobacco auxin-inducible gene	root tip	Van der Zaal *et al.* (1991)
β-tubulin	Root	Oppenheimer *et al.* (1988)
Tobacco root-specific genes	Root	Conkling *et al.* (1990)
B. napus G1-3b gene	Root	United States Patent No. 5, 401, 836
SbPRP1	Roots	Suzuki *et al.* (1993)
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
AtPRP4	leaves; flowers; lateral root primordia	http://salus.medium.edu/mmg/tierney/html
Seed-specific genes	Seed	Simon *et al.* (1985); Scofield *et al.* (1987); Baszczynski *et al.* (1990)
Brazil Nut albumin	seed	Pearson *et al.* (1992)
Legumin	Seed	Ellis *et al.* (1988)
Glutelin (rice)	Seed	Takaiwa *et al.* (1986);Takaiwa *et al.*

		(1987)
Zein	Seed	Matzke *et al.* (1990)
NapA	Seed	Stalberg *et al.* (1996)
Sunflower oleosin	seed(embryo and dry seed)	Cummins *et al.* (1992)
LEAFY	shoot meristem	Weigel *et al.* (1992)
Arabidopsis thaliana knat1	shoot meristem	Accession number AJ131822
Malus domestica kn1	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870
Stigma-specific genes	Stigma	Nasrallah *et al.* (1988); Trick *et al.* (1990)
Class I patatin gene	Tuber	Liu *et al.* (1991)
Blz2	Endosperm	EP99106056.7
PCNA rice	Meristem	Kosugi *et al* (1991); Kosugi and Ohashi (1997)

Table 3. Stress inducibl promot rs

Name	Stress	Reference
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang et al; Plant Science. Oct 28 1997; 129(1): 81-89
cor15a	Cold	Hajela et al., Plant Physiol. 93: 1246-1252 (1990)
cor15b	Cold	Wlihelm et al., Plant Mol Biol. 1993 Dec; 23(5):1073-7
cor15a (-305 to +78 nt)	cold, drought	Baker et al., Plant Mol Biol. 1994 Mar; 24(5): 701-13
rd29	salt, drought, cold	Kasuga et al., Nature Biotechnology, vol 18, 287-291, 1999
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	Heat	Barros et al., Plant Mol Biol, 19(4): 665-75, 1992. Marrs et al., Dev Genet.,14(1): 27-41, 1993. Schoffl et al., Mol Gen Gent, 217(2-3): 246-53, 1989.
smHSP (small heat shock proteins)	heat	Waters et al, J Experimental Botany, vol 47, 296, 325-338, 1996
wcs120	Cold	Ouellet et al., FEBS Lett. 423, 324-328 (1998)
ci7	Cold	Kirch et al., Plant Mol Biol, 33(5): 897-909, 1997 Mar
Adh	cold, drought, hypoxia	Dolferus et al., Plant Physiol, 105(4): 1075-87, 1994 Aug
pwsi18	water: salt and drought	Joshee et al., Plant Cell Physiol, 39(1): 64-72, 1998, Jan
ci21A	Cold	Schneider et al., Plant Physiol, 113(2): 335-45, 1997
Trg-31	Drought	Chaudhary et al., Plant Mol Biol, 30(6): 1247-57, 1996
Osmotin	Osmotic	Raghothama et al., Plant Mol Biol, 23(6): 1117-28, 1993

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Claims

1. A method for obtaining polynucleic acids comprising coding sequences and/or genes involved in environmental stress in plants, comprising the preparation of a cDNA library comprising coding sequences from siliques, introducing said coding sequences in yeast cells in a functional format and screening for polynucleic acids leading to an enhanced tolerance or resistance to environmental stress conditions in said transformed yeast cells.

2. An isolated polynucleic acid obtainable by a method according to claim 1.

3. The isolated polynucleic acid of claim 2 which encodes a polypeptide as listed in Table 1.

4. The isolated polynucleic acid of claim 3, which is chosen from:
   (a) any of SEQ ID NO 1, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77 or 121, or the complementary strands thereof;
   (b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;
   (c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b); or,
   (d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

5. The isolated polynucleic acid of any of claim 2 to 4, which encodes a plant homolog of yeast DBF2 kinase.

6. The isolated polynucleic acid of claim 5, which is chosen from:
   (a) SEQ ID NO 1, or the complementary strands thereof;
   (b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b); or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

7. Use of an isolated polynucleic acid of claims 2 to 3 which encodes an HSP 17.6A protein for the production of transgenic plants having an enhanced tolerance or resistance to environmental stress conditions.

8. Use of an isolated polynucleic acid of claim 7 for expression of the protein encoded thereby in a plant cell, with said polynucleic acid being chosen from:

(a) SEQ ID NO 3, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b); or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

9. Use of an isolated polynucleic acid as defined above which is chosen from:

(a) any of SEQ ID NO 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, or 119, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b) or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c),

for the production of transgenic plants having an enhanced tolerance or resistance to environmental stress conditions.

10. The isolated polynucleic acid of any of claims 2 to 4, which encodes a c74 protein which is chosen from:

(a) SEQ ID NO 5, or the complementary strand thereof;

(b) polynucleic acid sequences which hybridize to sequences defined in (a) or fragments thereof;

(c) polynucleic acid sequences which are degenerated as a result of the genetic code to the polynucleic acid sequences defined in (a) or (b); or,

(d) polynucleic acid sequences encoding a fragment of a protein encoded by a polynucleic acid of any one of (a) to (c).

11. An isolated polypeptide encoded by a polynucleic acid according to or as defined in any of claims 2 to 10, or a functional fragment thereof.

12. The isolated polypeptide of claim 11 having at least part of the sequence of any of SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, or 120.

13. A method for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising transiently introducing into a plant cell a recombinant DNA comprising a polynucleic acid of or as defined in any of claims 2 to 10 which is expressed in an amount effective to confer enhanced tolerance or resistance to environmental stress.

14. A method for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising stably introducing into a plant cell a recombinant DNA comprising a polynucleic acid of or as defined in any of claims 2 to 10 which is expressed in an amount effective to confer enhanced tolerance or resistance to environmental stress.

15. The method of claims 13 or 14 for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into said plant a polynucleic acid of claims 5 or 6 encoding a plant DBF2 kinase.

16. The method of claim 16 for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into said plant a polynucleic acid of claims 7 or 8 encoding an HSP 17.6A protein.

17. The method of claim 13 to 14 for producing a plant with enhanced tolerance or resistance to environmental stress, said method comprising introducing into said plant a polynucleic acid of claim 10 encoding a c74 protein.

18. The method of any of claims 13 to 17, comprising introducing into the genome of a plant cell one or more recombinant DNA molecules, said recombinant DNA molecules comprising:

- a polynucleic acid according to or as defined in any of claims 2 to 10, and,
- a plant expressible promoter, whereby said polynucleic acid is in the same transcriptional unit and under the control of said plant-expressible promoter.

19. A method for producing a plant with enhanced tolerance or resistance to environmental stress, comprising introducing into the genome of a plant cell one or more recombinant DNA molecules, said recombinant DNA molecules comprising:

- a DNA encoding a protein which when expressed in said plant cell at an effective amount indirectly increases or induces the expression of an endogenous polynucleic acid according to or as defined in any of claims 2 to 10 or indirectly increases or induces the activity of a polypeptide of claims 11 or 12, and,

- a plant expressible promoter, whereby said DNA is in the same transcriptional unit and under the control of said plant-expressible promoter.

20. A method of claim 19 wherein said DNA encodes a sense or antisense RNA molecule or a ribozyme capable of increasing or inducing the expression of said endogenous polynucleic acid sequence according to or as defined in any of claims 2 to 10.

21. A recombinant polynucleic acid comprising:
a polynucleic acid according to or as defined in any of claims 2 to 10, and,
a plant expressible promoter, whereby said polynucleic acid is in the same transcriptional unit and under the control of said plant-expressible promoter.

22. A recombinant polynucleic acid comprising:
(a) a DNA encoding a protein which when expressed in said plant cell at an effective amount increases or induces the expression of an endogenous polynucleic acid according to or as defined in any of claims 2 to 10 or increases or induces the activity of a polypeptide of claims 11 or 12, and,
(b) a plant expressible promoter, whereby said DNA is in the same transcriptional unit and under the control of said plant-expressible promoter.

23. The recombinant polynucleic acid of claim 22, wherein said DNA encodes an anti-sense RNA, a ribozyme or a sense RNA which when expressed in a cell of a plant increases or induces the expression of an endogenous polynucleic acid according to or as defined in any of claims 2 to 10 or which induces or increases the activity of a protein of claim 11 or 12.

24. The recombinant polynucleic acid of claim 21 comprising at least part of the nucleotide sequence of any of SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 90, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, or 121, or part thereof.

25. The recombinant polynucleic acid of claim 21 to 24 comprising at least part of the coding sequence of a gene encoding a protein as listed in Table 1.

26. The recombinant polynucleic acid of any of claims 21 to 25 wherein said plant-expressible promoter is a constitutive promoter.

27. The recombinant polynucleic acid of any of claims 21 to 25 wherein said plant-expressible promoter is a stress-inducible or organ- or tissue-specific promoter.

28. The recombinant polynucleic acid of any of claims 21 to 26 wherein said plant-expressible promoter is the 35S promoter of CaMV.

29. A recombinant host cell transformed with at least one isolated polynucleic acid of or as defined in any of claims 2 to 10.

30. A plant cell transformed with a recombinant polynucleic acid of any one of claims 21 to 28.

31. A plant consisting essentially of plant cells of claim 30.

32. A callus consisting essentially of plant cells of claim 30.

33. A harvestable part, organ, tissue or propagation material of a plant of claim 31, comprising said recombinant DNA.

34. The use of a recombinant polynucleic acid of claim 21 to 28 to produce transgenic plants.

35. A probe which is part of the polynucleic acid sequence of or as defined in any of claims 2 to 10 and which hybridizes specifically with said polynucleic acid or the complement thereof.

36. A primer which is part of the polynucleic acid sequence of or as defined in any of claims 2 to 10 and which specifically amplifies said polynucleic acid or the complement thereof.

37. A composition comprising a polynucleic acid sequence of or as defined in any of claims 2 to 10, a polypeptide of claim 11 or 12, a probe of claim 35 or a primer of claim 36.

FIGURE 1 A

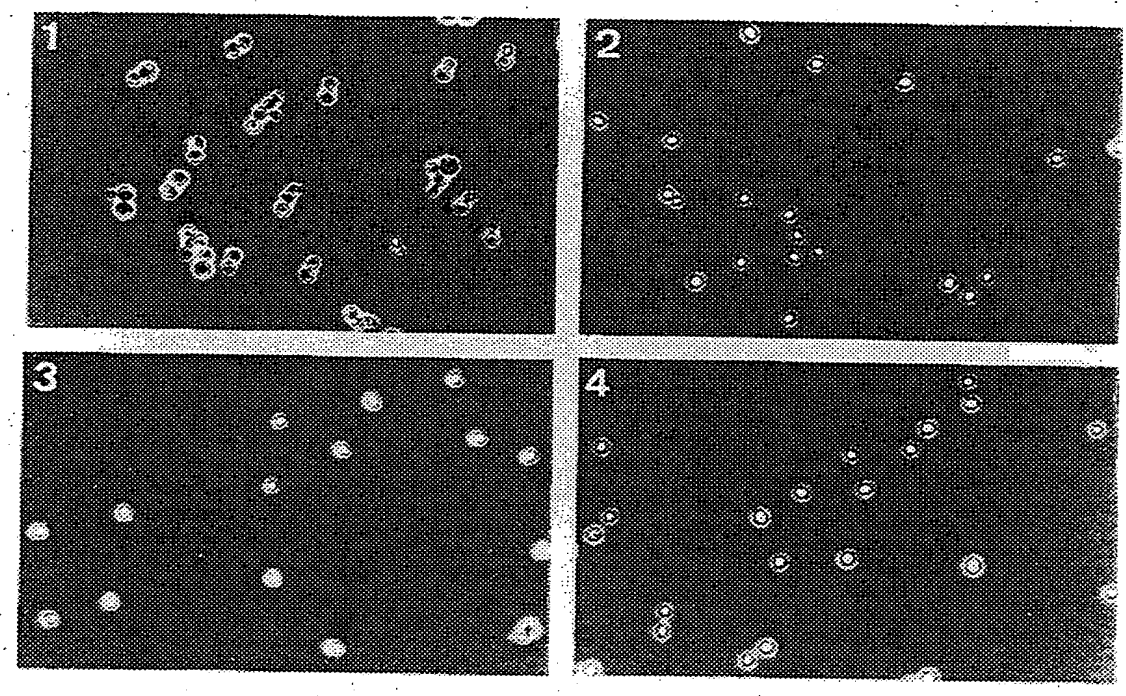


FIGURE 1B

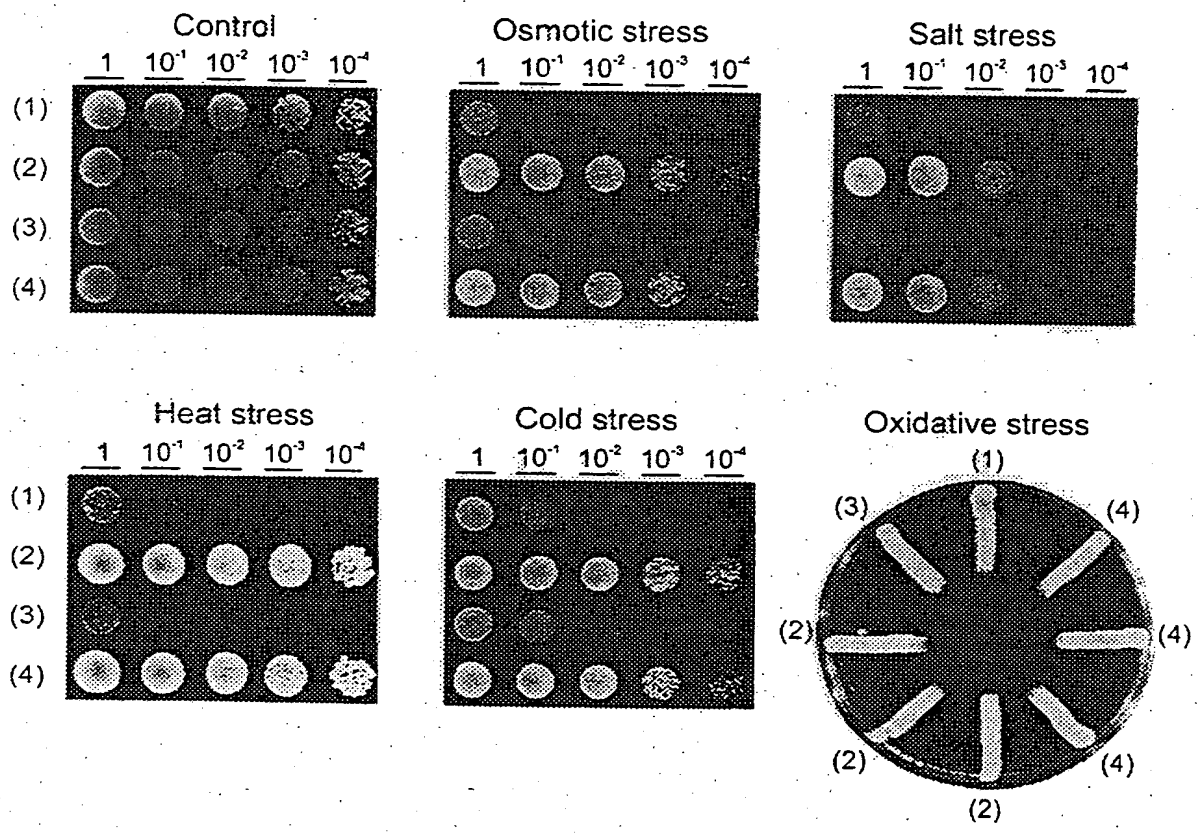


FIGURE 2

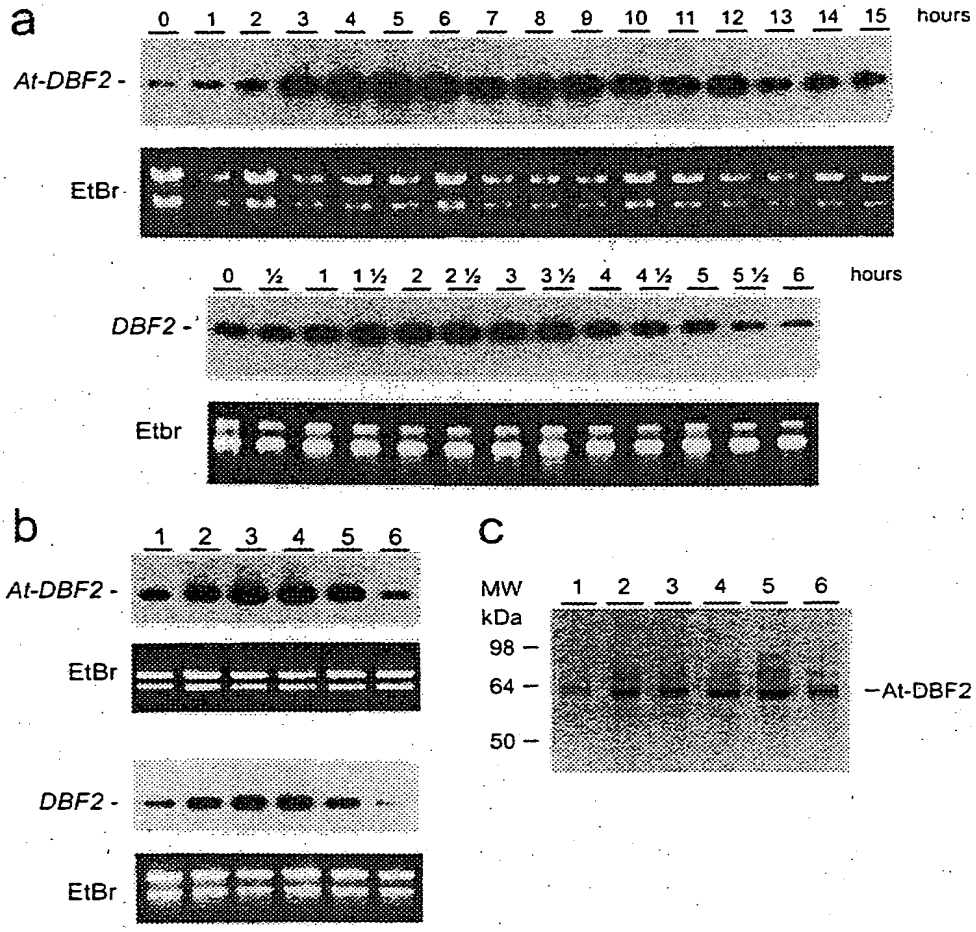


FIGURE 3

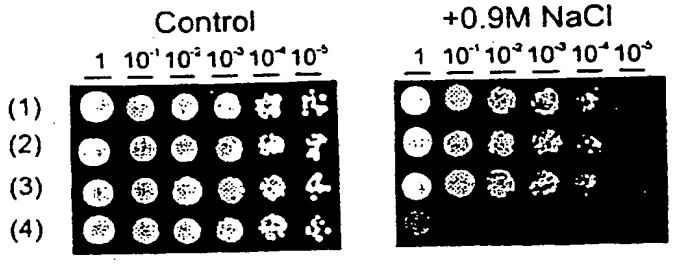
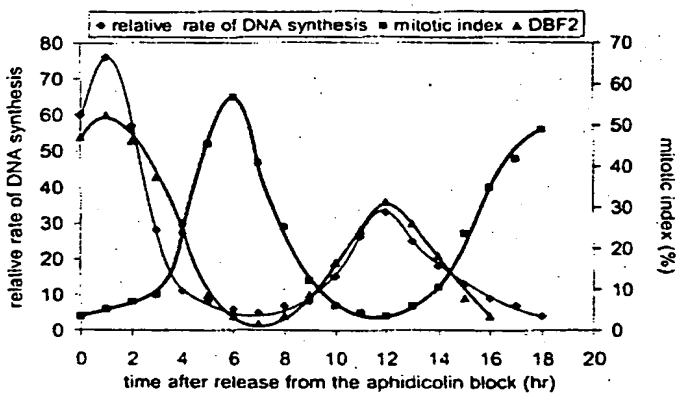
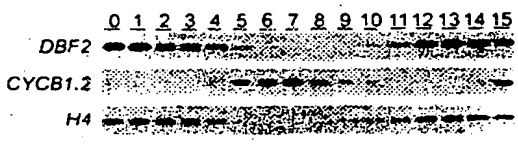


FIGURE 4

a



b



c

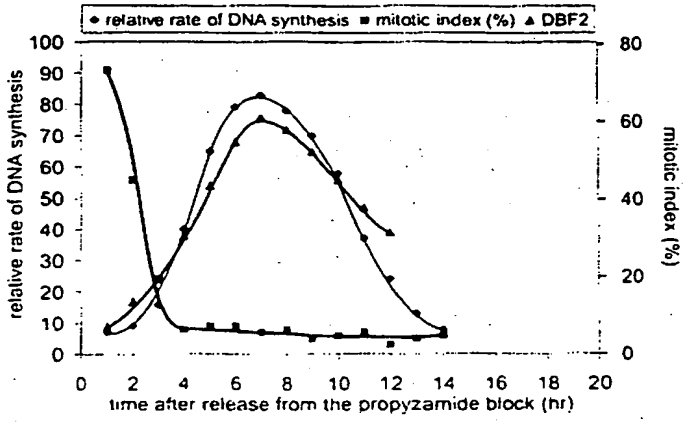


FIGURE 5

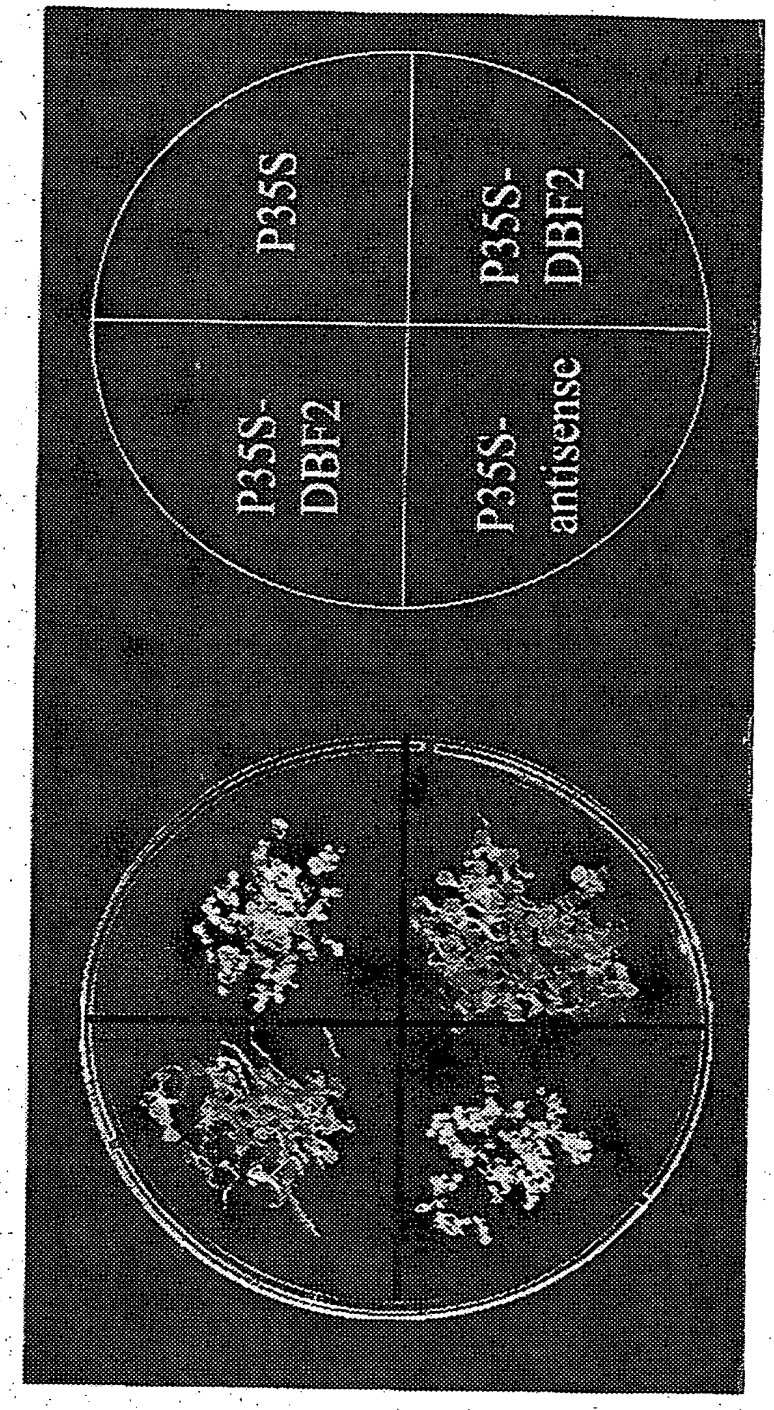


FIGURE 6

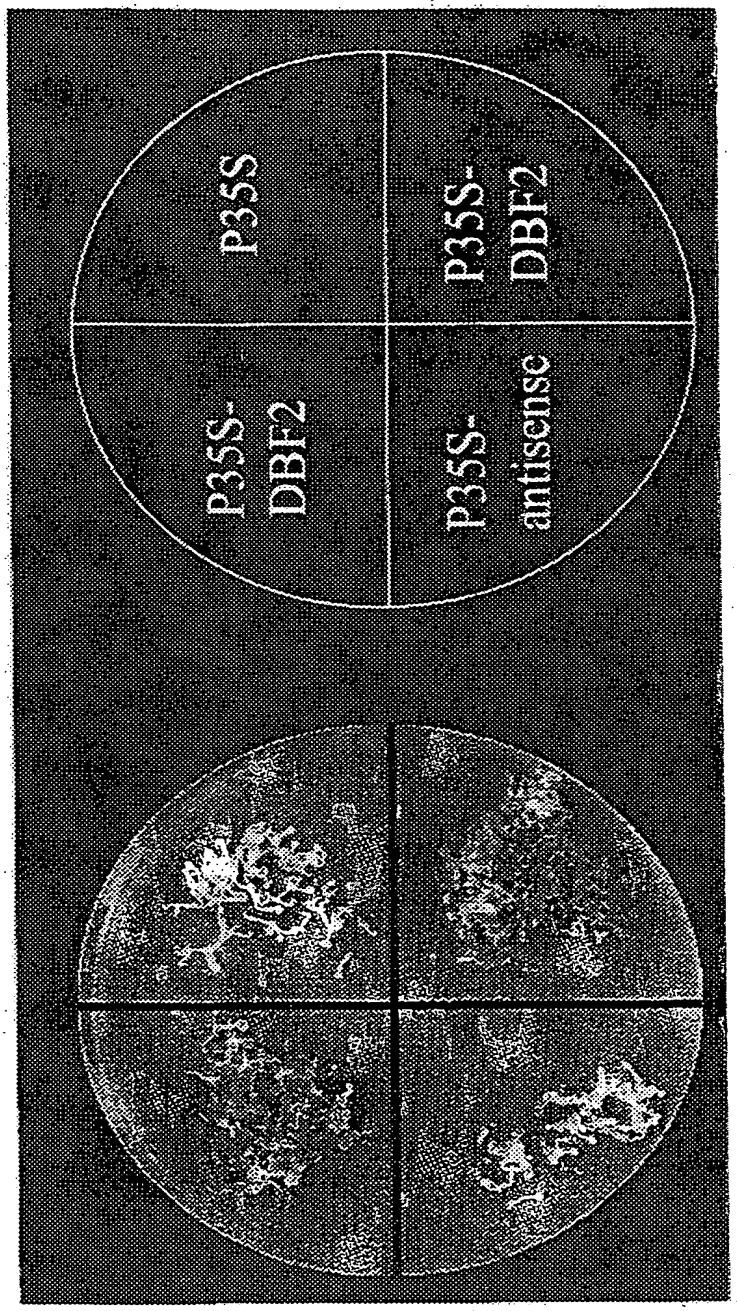


FIGURE 7

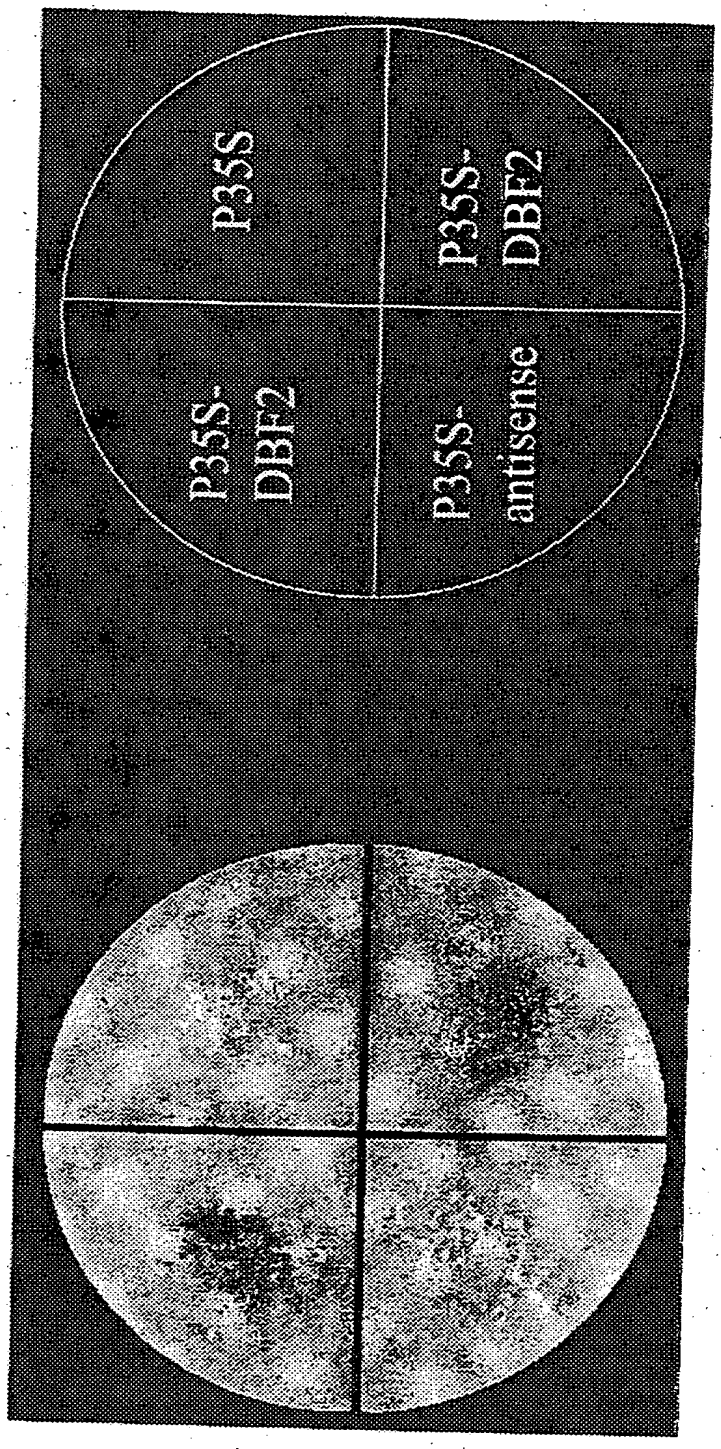


FIGURE 8

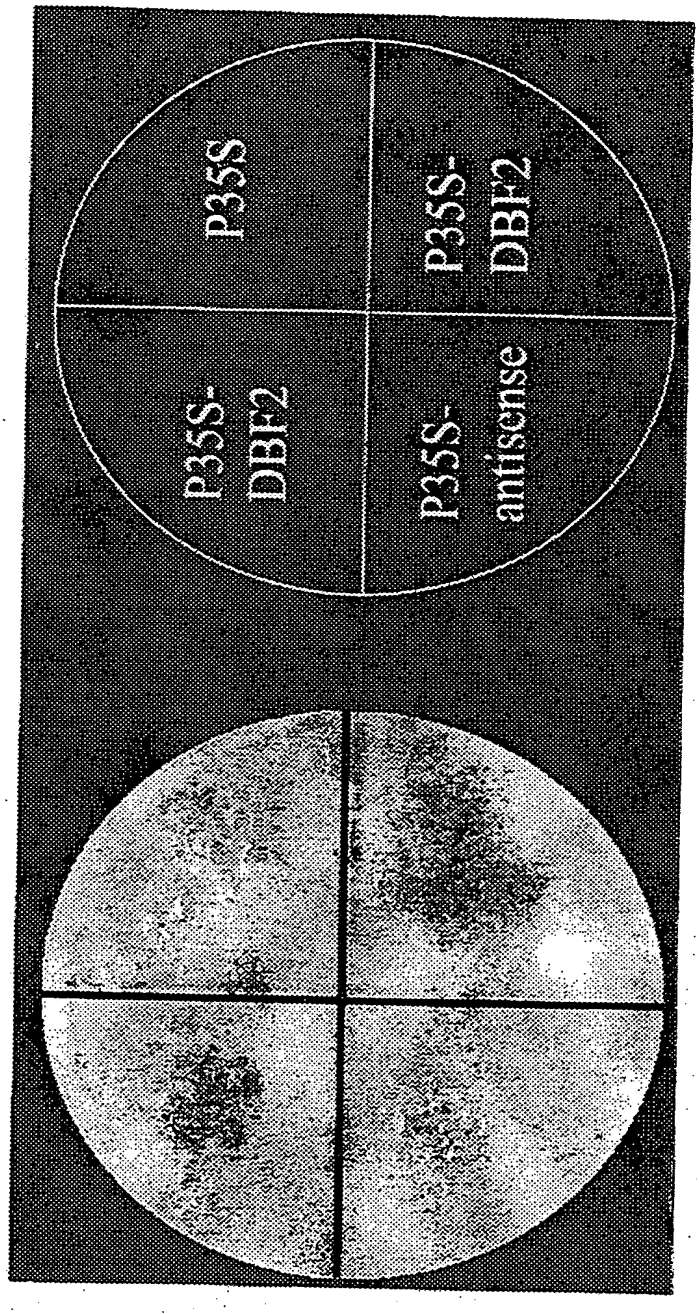


FIGURE 9

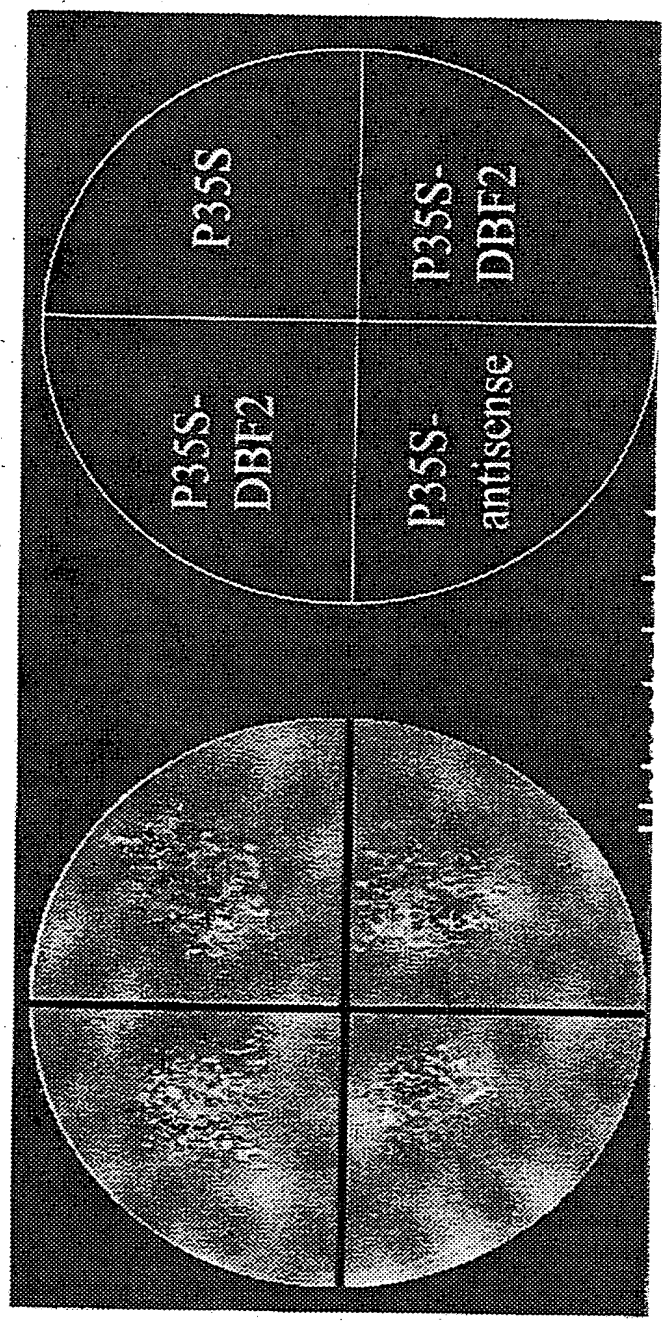


FIGURE 10

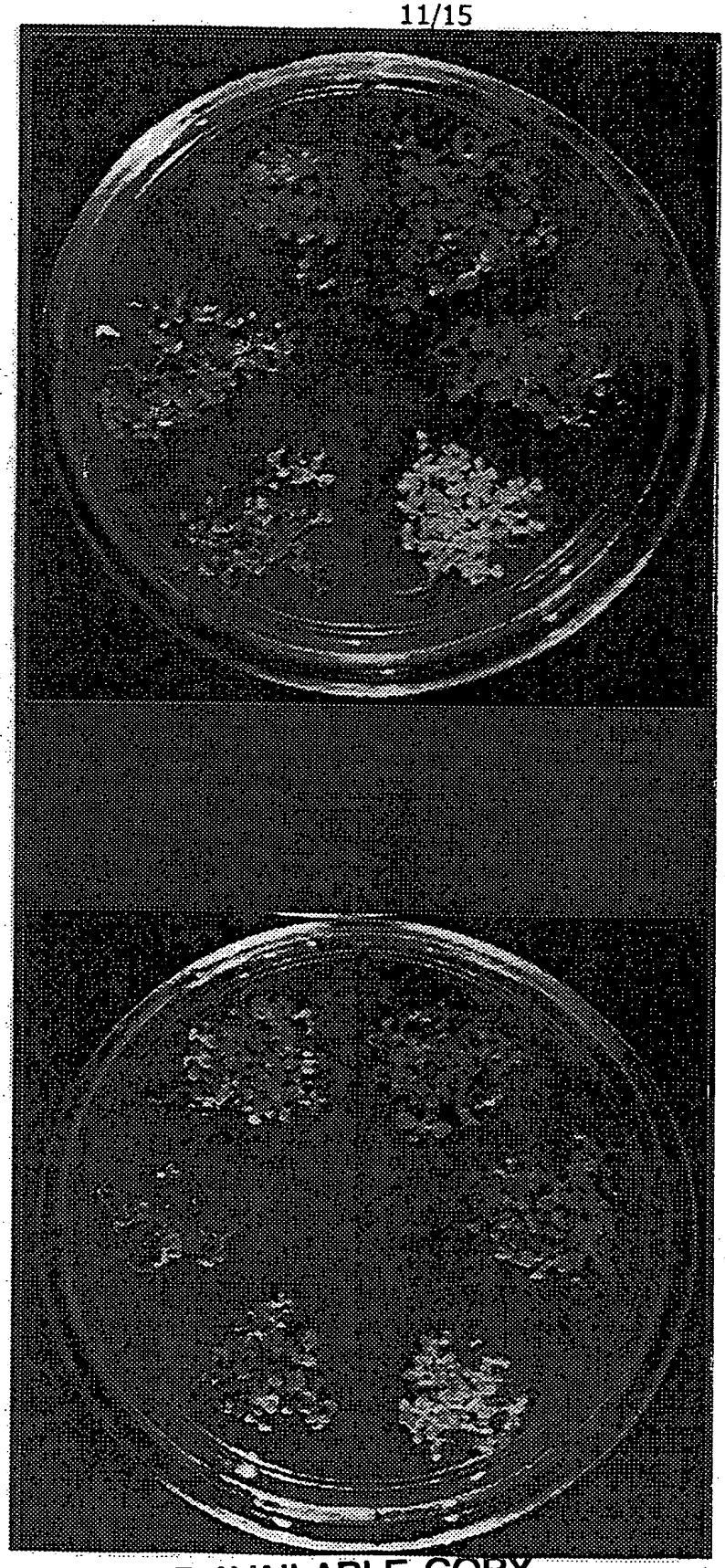


FIGURE 11

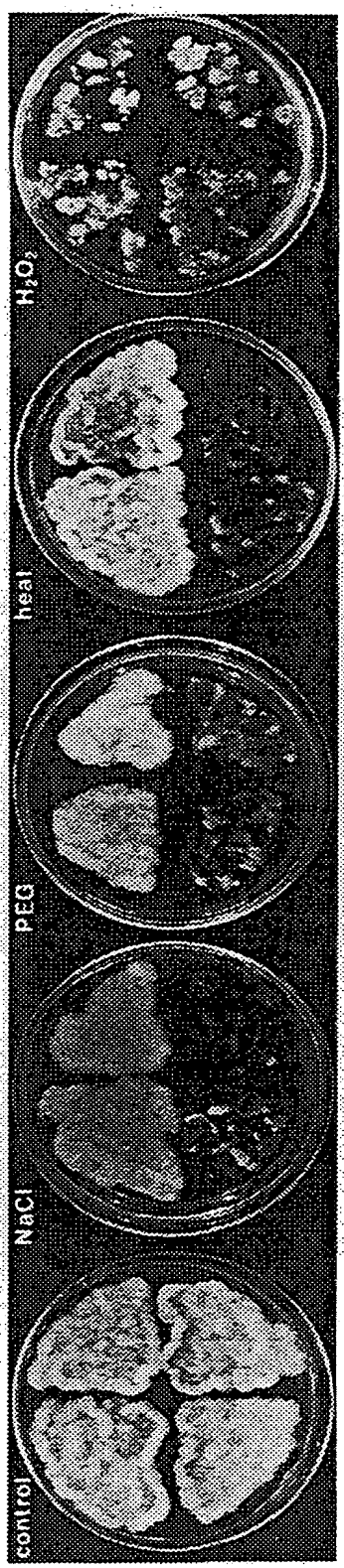


FIGURE 12A

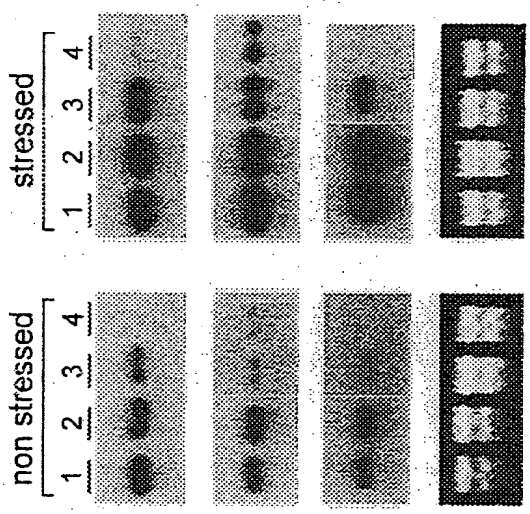


FIGURE 12c

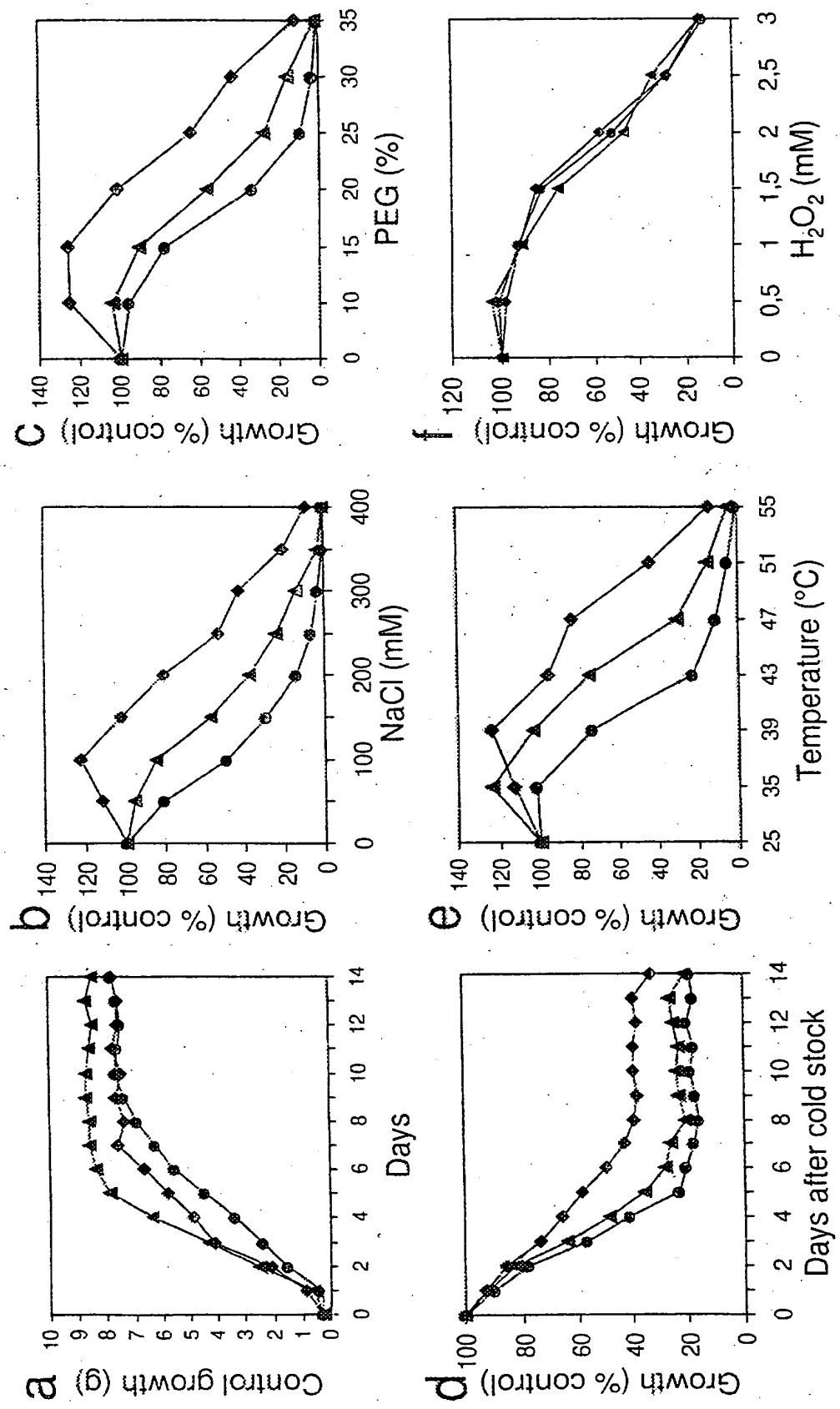


FIGURE 12B

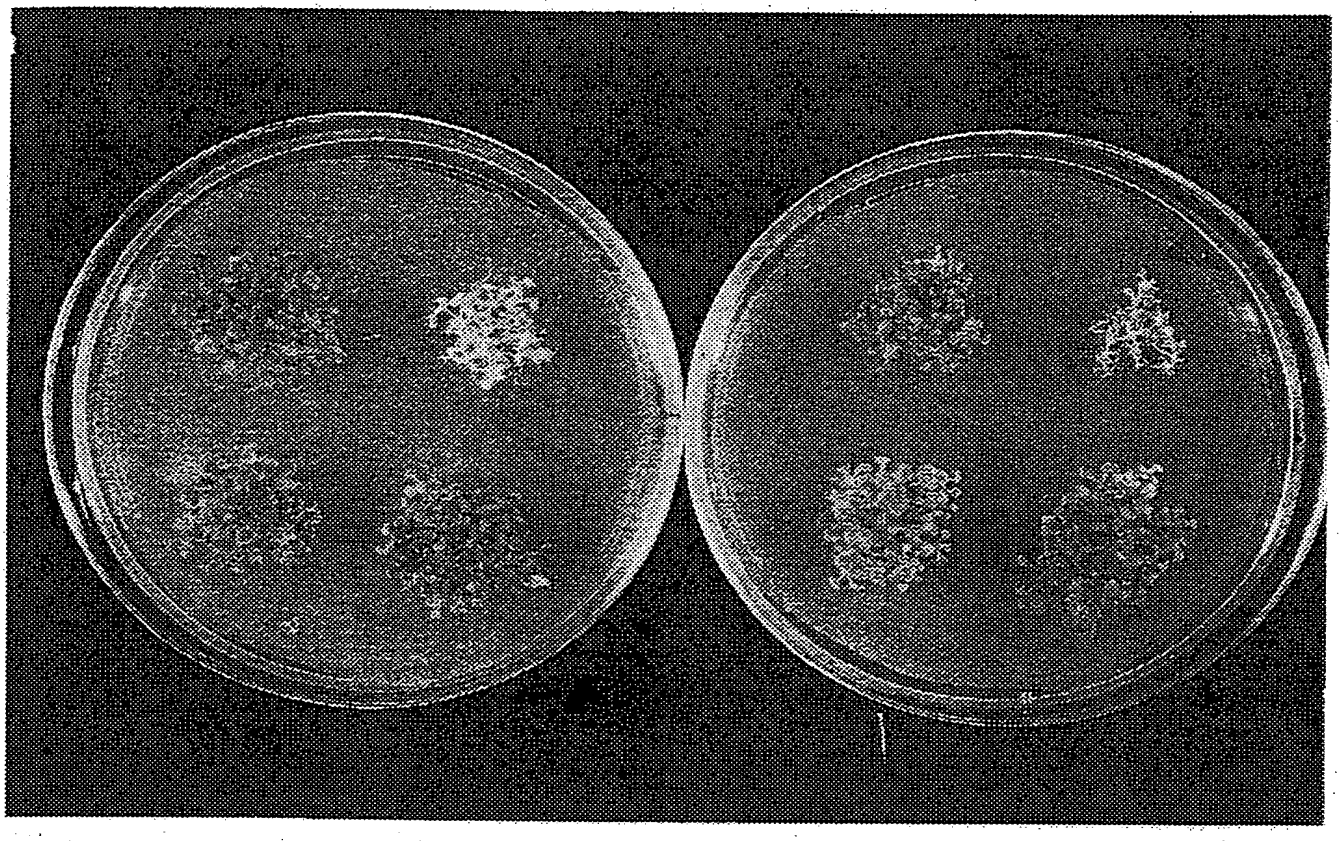


FIGURE 13

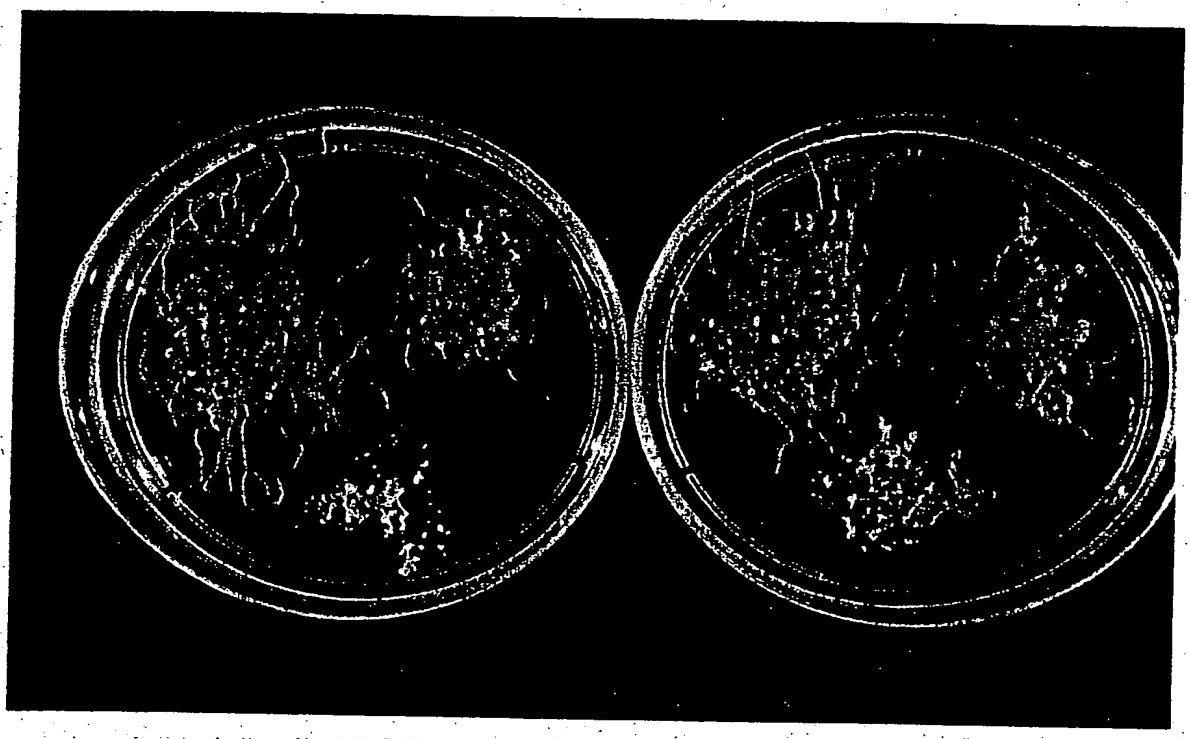


FIGURE 14